The effect of extracellular pH upon regulation of glucose metabolism in Saccharomyces cerevisiae.

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THE EFFECT OF EXTRACELLULAR pH UPON REGULATION OF GLUCOSE METABOLISM IN *Saccharomyces cerevisiae*

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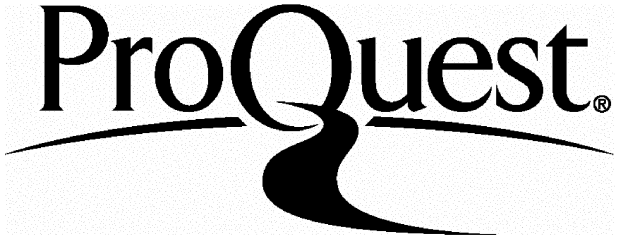

A C K N O W L E D G E M E N T S

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TO MY FATHER AND MOTHER

C O N T E N T S

continued-

SUMMARY

1. The effect of extracellular pH upon the regulation of aerobic glucose metabolism in resting cells of Saccharomyces cerevisiae was investigated using stationary phase cells suspended in sodium phosphate buffers at pH 2.0, pH 4.5 and pH 7.5.

2. It was found that the activity of phosphofructokinase (EC 2.7.1.11) varied with the pH of the environment being higher in cells suspended at pH 2.0 and pH 4.5 than at pH 7.5. Although the possibility of changes in the intracellular pH causing this effect could not be excluded completely, the differences in the activity of phosphofructokinase, between the three pH values, could be correlated with differences in the intracellular content of adenine nucleotides and citric acid. In addition to this effect upon phosphofructokinase, at pH 2.0 the activity of the glucose transport system was reduced by extracellular pH.

3. At pH 2.0 the combination of the high activity of phosphofructokinase and low activity of the glucose transport system reduced substrate supply to the polysaccharide synthesising system, so that more glucose was degraded via glycolysis and less synthesised to polysaccharide than at pH 4.5 and pH 7.5.

At pH 7.5 compared to pH 4.5, a lower activity of phosphofructokinase reduced the amount of glucose which was degraded via glycolysis, and increased the amount synthesised to polysaccharide.

4. In vivo evidence was obtained that, during glucose utilisation, the activity of pyruvate kinase (EC 2.7.1.40) may be regulated by the intracellular content of fructose-1,6-diphosphate, which in turn was regulated by the activity of phosphofructokinase.

5. From experiments with cells suspended at pH 4.5 and pH 7.5 in vivo evidence was obtained to support the following regulatory functions of phosphofructokinase in the aerobic glucose metabolism of resting cells of Saccharomyces cerevisiae.

   a) That it was the rate controlling enzyme within the glycolysis system and therefore regulated the rate of glycolysis.

   b) That via the intracellular content of hexose monophosphate it regulated the activity of the glucose transport system and hence the rate of glucose uptake.

   c) That via the activating effect of fructose-1,6-diphosphate upon pyruvate kinase it co-ordinated the activity of enzymes within the glycolysis system.

   d) That via alterations in the activity of the glycolysis system it altered the relative activities of the glycolysis and polysaccharide synthesising systems and hence controlled the metabolic fate of incorporated glucose.

A B B R E V I A T I O N S

INTERMEDIATES

HMP	hexose monophosphate (glucose-6-phosphate + fructose-6-phosphate)
G6P	glucose-6-phosphate
F6P	fructose-6-phosphate
FDP	fructose-1,6-diphosphate
TP	triosephosphate (dihydroxyacetonephosphate + glyceraldehyde-3-phosphate)
DHAP	dihydroxyacetonephosphate
GAP	glyceraldehyde-3-phosphate
PGA	phosphoglyceric acid (3-phosphoglyceric acid + 2-phosphoglyceric acid)
3PG	3-phosphoglyceric acid
2PG	2-phosphoglyceric acid
PEP	phosphoenolpyruvate

ENZYMES

HK,	hexokinase, ATP:D-hexose 6-phosphotransferase (E C 2.7.1.1)
PFK,	phosphofructokinase, ATP:D-fructose-6-phosphate 1-phosphotransferase (E C 2.7.1.11)
GAPDH,	glyceraldehydephosphate dehydrogenase, D-glyceraldehyde-3-phosphate:NAD oxidoreductase (E C 1.2.1.12)
PGK,	phosphoglycerate kinase, ATP:3-phospho-D-glycerate 1-phosphotransferase (E C 2.7.2.3)
PK,	pyruvate kinase, ATP:pyruvate phosphotransferase (E C 2.7.1.11)

ADENINE NUCLEOTIDES

ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
NAD+	nicotinamide-adenine dinucleotide,oxidised form
NADH+H+	nicotinamide-adenine dinucleotide,reduced form
NADP+	nicotinamide-adenine dinucleotide phosphate, oxidised form

I N T R O D U C T I O N

(a) THE EFFECT OF EXTRACELLULAR pH UPON THE GLUCOSE METABOLISM OF SACCHAROMYCES CEREVISIAE.

In the absence of potassium the rate of glucose metabolism, by resting cells of Saccharomyces cerevisiae, is markedly effected by extracellular pH. Thus Rothstein (1954) found that the pH - activity curve for anaerobic glucose uptake and fermentation was biphasic with optima at pH 5.0 and pH 8.0 and also reported a similar effect upon aerobic glucose uptake and respiration.

A similar biphasic pH-activity curve for all fermentable hexoses was found by Van Steveninck and Dawson (1968) though the precise position of the optima was found to depend upon both the yeast strain and the hexose being metabolised. Under conditions of reduced glucose uptake no significant accumulation of intracellular free hexose or of intermediates could be detected and thus it was concluded that the extracellular pH must change the activity of the glucose transport system. This effect in turn has been argued to be due to H^+ions changing the conformation of certain polyphosphate chains, essential for the phosphorylation of a carrier, involved in active glucose transport (Rothstein and Van Steveninck, 1966). The depressant effect of H^+ions upon glucose uptake, fermentation and respiration can be completely reversed by the presence of potassium in the extracellular medium (Rothstein, 1954) which appears to act competitively by displacing H^+ions from carrier sites for which the two ions compete. (Van Steveninck and Dawson, 1968).

That H^+ions do in fact limit the activity of the glucose transport system is supported by work in this laboratory where it has been found that an increased rate of glucose uptake and glycolysis, in the presence of potassium, is associated with a higher intracellular

content of HMP (J.Ottery, personnel communication, 1969). In addition the presence of potassium appears to maintain a lower energy status (ATP/ADP + AMP) of the cells, which would be expected to contribute towards the higher rate of glycolysis (Ramaiah, Hathaway and Atkinson, 1964) observed in the presence of potassium. Other workers (Pena et al, 1969) have shown that with cells metabolising glucose in a potassium free medium, the addition of potassium decreases the intracellular content of ATP and increases that of ADP, as well as increasing the rate of glycolysis. Thus it appears that extracellular pH may effect glucose metabolism directly by altering the rate of glucose transport and possibly indirectly by altering the energy status of the cells.

Besides the effect of extracellular pH upon the rate of glucose metabolism others have been reported concerning the metabolic fate of incorporated glucose and these effects appear to be independent of potassium. Thus Neuberg first observed that the formation of glycerol and acetic acid was increased and that of ethanol decreased at alkaline pH. Similar results were obtained by Neish and Blackwood (1951) and Rothstein (1954). This latter worker found that over the pH range 8.0 to 10.0 a marked inhibition of glycogen synthesis occurred. Therefore in this instance extracellular pH influenced the distribution of glucose between synthetic (glycogen) and degradative (glycerol) end products. Sussman, Speigleman and Reiner (1947) found that CO_2 production was increased at pH 8.5 compared to pH 4.5, which was interpreted as being due to a decreased glucose assimilation into polysaccharide at alkaline pH.

Other workers have reported an effect of extracellular pH upon polysaccharide synthesis which conflicts with those described above.

Thus Wiggins et al. (1952) found that glycogen synthesis was increased and CO_2 production decreased at pH 8.5 compared to pH 4.5 in both the presence and absence of potassium. In addition Trevelyan (Personnel communication, 1969) found that at low pH (2.2-3.1) the synthesis of a trichloroacetic acid soluble carbohydrate (trehalose) was markedly reduced compared to any effect upon glucose uptake and fermentation, with cells suspended in buffers containing potassium.

The discrepancy between the reported effects of extracellular pH upon polysaccharide synthesis ie. whether this is decreased (Rothstein, 1954) or increased (Wiggins et al. 1952; Trevelyan, 1969) at alkaline pH, may be due to the use of different yeast strains. Wiggins et al. (1952) and Trevelyan (1969) used a bakers yeast as supplied by D.C.L. Ltd., Epsom, England which was also the source of the yeast used in the work reported in this thesis.

The effect of extracellular pH upon the rate of glucose metabolism (Rothstein, 1954) may be satisfactorily explained in terms of an effect upon the activity of the glucose transport system (Van Steveninck and Dawson, 1968). However, the manner in which the distribution of incorporated glucose is influenced by extracellular pH is not so clear. This may be attributed to two factors:- 1. The uncertainty as to whether extracellular pH effects the intracellular pH of the yeast cell. 2. Investigations into the effect of extracellular pH upon the regulation of intermediary metabolism have not been reported.

Those investigations concerned with the effect of extracellular pH upon the intracellular pH of the yeast cell have been carried out by two different procedures which give conflicting results. An

indirect procedure used in early investigations involves the measurement of the pH of cell extracts prepared from cells suspended at different pH values. Such investigations suggest that extracellular pH does not effect the intracellular pH of resting yeast cells (Tait and Fletcher, 1936; Drews, 1926). However, this method does not necessarily represent the in vivo conditions established whilst cells are actually exposed to a particular pH value. Also the pH of freshly prepared yeast extracts is known to decrease spontaneously (Conway and Downey 1950a; Kotyk, 1963). In order to overcome these difficulties Kotyk (1963) determined intracellular pH directly by measuring the distribution of bromothymol blue between yeast cells and the extracellular medium. Using this method a marked effect of extracellular pH (pHo) upon intracellular pH (pHi) was observed. Thus, in resting cells pHi changed rapidly from pH 5.8 to pH 7.6 between pHo values of 5.0-7.0, but either side of these pHo values pHi was maintained constant (Kotyk, 1963).

The available evidence as to the effect of extracellular pH upon intracellular pH during active glucose metabolism is even more conflicting than that for resting cells. Thus Kotyk (1963) found that with cells suspended in distilled water pHi decreased from pH 7.5 to pH 6.5, but was maintained constant by cells suspended in 0.1MKCl even though pHo decreased from pH 7.0 to pH 3.5 in both cases. These results suggest that potassium enables the cell to maintain its internal pH. However, Conway and Downey (1950a) found that the pH of extracts prepared from cells fermenting glucose in 0.1MKCl increased from pH 5.80 to pH 6.35, whilst at the same time the pH of an "outer metabolic" region decreased to below pH 4.2. In contrast to those investigations which suggest that intracellular pH may change during fermentation, Rothstein (1954) has reported that the pH of extracts prepared from

cells fermenting glucose, in potassium free media over the pH range pH 1.7-11.0, was unaltered.

It is therefore difficult to draw conclusions as to the effect of extracellular pH upon the intracellular pH of the yeast cell, although the results obtained by Kotyk (1963) may be regarded as being most likely to represent the in vivo condition.

The effect of extracellular pH upon the distribution of incorporated glucose might be due either to an effect of pH upon the activities of polysaccharide synthesising and glucose degrading systems or indirectly to a change in regulatory mechanisms. Rothstein (1954) concluded that extracellular pH effected the activity of enzymes in or very near to the pH sensitive "outer metabolic" region reported by Conway and Downey (1950b). This "outer metabolic" region was identified by the latter authors with the cell wall and outer surface of the cell membrane and some evidence has been obtained that the glycolytic enzymes are contained within an insoluble structural unit (Rothstein et al. 1959).

However, the possibility that extracellular pH might effect regulatory mechanisms was suggested by some results obtained by Brunt and Stewart (1967). These authors found that at acid pH polysaccharide synthesis and glycolysis were inhibited in a manner which was not simply related to an effect upon glucose uptake. This present investigation was undertaken to determine whether an effect of extracellular pH upon polysaccharide synthesis and glucose degradation was related to changes in known regulatory mechanisms in yeast.

(b). REGULATORY ENZYMES CONCERNED WITH POLYSACCHARIDE SYNTHESIS AND AEROBIC GLUCOSE DEGRADATION IN SACCHAROMYCES CEREVISIAE.

b1. CRITERIA USED TO IDENTIFY REGULATORY ENZYMES.

A regulatory enzyme is defined as one the activity of which is controlled by factors other than substrate availability and which controls the rate of flux or the concentrations of intermediates of a metabolic pathway (Newsholme and Gevers, 1967). Regulatory enzymes usually possess the lowest activity of those enzymes in a metabolic pathway (Krebs, 1963) and are likely to catalyse nonequilibrium reactions so that the extent to which their activity need be changed for control purposes is less than that for high activity enzymes. Indeed it has been argued that those enzymes which catalyse equilibrium reactions cannot control the rate of flux along a metabolic pathway (Hess, 1963; Bücher and Russmann, 1964; Hess and Brand, 1965a) since their activity is controlled by substrate availability.

A number of experimental procedures are commonly used and generally accepted as being valid for the identification of potential regulatory enzymes. Only those relative to studies upon regulatory enzymes concerned with polysaccharide synthesis and aerobic glucose degradation, in yeast, will be considered here.

1. MAXIMAL ENZYME ACTIVITIES IN VITRO.

Low activity and therefore potential regulatory enzymes may be identified by measuring the maximal catalytic activity of the enzymes in a metabolic pathway. This procedure is generally valid since, although inactivation may occur during extraction and *in vitro* assay conditions may not represent those operating *in vivo*, it is known from general experience that enzymes of regulatory importance usually possess activities which are ten to one hundred fold lower than nonregulatory enzymes (Krebs, 1963).

2. ENZYMES CATALYSING NONEQUILIBRIUM REACTIONS IN VIVO.

Those enzymes which catalyse nonequilibrium reactions in vivo may be identified by measuring the intracellular content of all the intermediates and cofactors of a metabolic pathway and calculating the mass action ratio for each reaction. If the calculated mass action ratio is much smaller than the equilibrium constant for a particular enzyme reaction this indicates that the enzyme catalyses a nonequilibrium reaction in vivo and therefore is a potential regulatory enzyme.

3. EFFECTS OF ALTERED FLUX RATES ON THE INTRACELLULAR CONTENT OF INTERMEDIATES.

Those enzymes which catalyse nonequilibrium reactions in vivo and are in fact involved in metabolic regulation may be identified in vivo by measuring the level of intermediates of a metabolic pathway under two or more conditions where the overall flux rate is different. Thus, if the substrate concentration changes in an opposite sense to the flux rate this is evidence for a regulatory enzyme (Krebs, 1957; Krebs and Kornberg, 1957). Generally changes in the level of products are irrelevant to the metabolic regulation of an enzyme catalysing a nonequilibrium reaction (Newsholme and Gevers, 1967). However, if the level of intermediates prior to an enzyme step changes in an opposite sense to those subsequent to it, this clearly identifies the rate controlling step within a metabolic pathway (Gosh and Chance, 1964).

4. DETERMINATION OF THE PROPERTIES OF AN ENZYME IN VITRO.

Once an enzyme has been identified as being regulatory it should be isolated and its properties investigated in order that a theory of metabolic regulation may be formulated and tested. Often this process is carried out in reverse ie, the properties of an enzyme are

investigated *in vitro* to determine whether they comply with those generally accepted for regulatory enzymes viz, the activity of the enzyme is controlled by factors other than substrate availability.

5. OTHER PROCEDURES USED TO IDENTIFY REGULATORY ENZYMES IN YEAST.

In addition to those already described other procedures have been used to identify regulatory enzymes in yeast, especially those concerned with the regulation of glycolysis. These procedures have been concerned with changes in the level of intermediates and cofactors during oscillating glycolysis in whole cells (Hommes,1964) and cell extracts (Chance, Hess and Betz, 1964). Also the effect of pure enzymes (Hess, Brand and Cassuto, 1965) and intermediates and cofactors (Chance, Schoener and Elsaesser, 1965).upon the characteristics of oscillations in cell free extracts has been used to identify regulatory enzymes.

b2. ENZYMES CONCERNED WITH POLYSACCHARIDE SYNTHESIS AND AEROBIC GLUCOSE DEGRADATION WHICH HAVE BEEN IDENTIFIED AS BEING REGULATORY.

Measurements of the activities of glycolytic enzymes in cell extracts have shown that PFK and PK are present in the lowest activity (Hess and Brand, 1965a). These two enzymes are ideally suited to a regulatory role since they catalyse essentially irreversible reactions (Krebs and Kornberg, 1957) which are therefore likely to be displaced far from equilibrium *in vivo* (Meyerhof, 1944). Although a detailed analysis of the mass action ratio for reactions catalysed by glycolysis enzymes in yeast does not appear to exist in the literature, it can be calculated from results presented by a number of workers that both PFK (Hommes, 1964; Betz and Chance, 1964; Betz and Moore, 1967) and PK (Hommes, 1964; Hommes, 1966) do in fact catalyse nonequilibrium reactions *in vivo*.

In vivo measurements of the intracellular content of intermediates, in yeast, under conditions of different steady state flux rates have been limited to studies of the Pasteur effect. Thus the decreased glycolysis rate under aerobic compared to anaerobic conditions is associated with a higher level of HMP (Lynen et al. 1959; Salas et al, 1965) which indicates that PFK limits the rate of glycolysis under aerobic conditions. In addition to PFK both PGK and PK have been reported to be involved in the regulation of the Pasteur effect in yeast (Pye, 1965).

Under aerobic conditions the rate of glucose uptake, as well as the glycolysis rate, is lower than under anaerobic conditions (Lynen et al. 1959). Since yeast hexokinase is not inhibited by G6P (DelaFuente and Sols, 1964) the glucose transport system must be considered as a regulatory step in glucose metabolism (Sols, 1968).

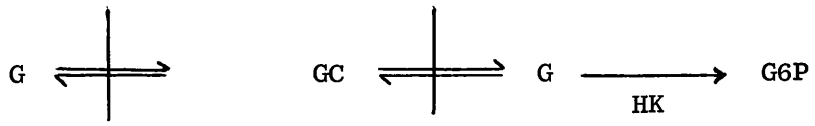
Studies upon oscillating glycolysis in whole cells have also indicated that PFK is a regulatory enzyme *in vivo*, since an increase in the glycolysis flux rate is associated with a decrease in HMP and vice versa (Gosh and Chance, 1964; Betz and Chance, 1964). Studies upon the mechanism of oscillating glycolysis have shown that glycolysis enzymes other than PFK are regulatory *in vivo*. Thus analysis of the phase relationship (Gosh and Chance, 1964) of intermediates and flux rates in whole cells (Hommes, 1964; Betz and Chance 1964) and titration studies where the characteristics of the oscillations are altered by addition of pure enzymes. (Hess, Brand and Cassuto, 1965; Hess and Brand, 1965b; Hess, 1965a) and intermediates (Chance, Schoener and Elsaesser, 1965; Chance, Hess and Betz, 1964) to cell free extracts, have indicated that the GAPDH/PGK system and PK are regulatory.

In vivo evidence for regulatory enzymes concerned with glucose metabolism, in yeast, is largely limited to the glycolysis enzymes. Evidence for regulatory enzymes concerned with polysaccharide synthesis and aerobic glucose degradation has been obtained from in vitro studies with isolated enzymes. Such investigations have shown that glycogen synthetase (Rothman and Cabib, 1967) citrate synthetase (Parvin and Atkinson, 1968) and NAD^+ isocitrate dehydrogenase (Atkinson, 1968) are regulatory enzymes since their activity is controlled by factors other than substrate availability.

b3. PROPERTIES OF REGULATORY ENZYMES CONCERNED WITH POLYSACCHARIDE SYNTHESIS AND AEROBIC GLUCOSE DEGRADATION.

GLUCOSE TRANSPORT SYSTEM.

The first step in glucose utilisation by S.cerevisiae involves transport across the cell membrane by an active carrier mediated process (Kleinzeller and Kotyk, 1968) after which the glucose is phosphorylated by hexokinase.



A modification of this basic model involves a phosphorylated carrier which combines with glucose. The resulting glucose - carrier - phosphate complex is then argued to react with hexokinase yielding free G6P and free carrier (Rothstein and Van Steveninck, 1966).

Since free glucose does not normally accumulate inside the yeast cell (Kleinzeller and Kotyk, 1968; Sols, 1968) it has been proposed that hexokinase is present in excess and that the transport system is feedback regulated by G6P (Sols, 1968). This hypothesis is supported by several kinds of evidence from in vivo studies. Thus a lower rate of glucose utilisation under aerobic conditions is associated with a

higher content of G6P compared to anaerobic conditions (Lynen et al, 1959; Salas et al, 1965). Also the rate of glucose utilisation during oscillating glycolysis decreases as G6P increases and vice versa (Betz and Hinrichs, 1968). In addition, the rate of uptake of xylose and L-sorbose is inhibited under aerobic compared to anaerobic conditions when glucose is also present in the medium and being transported into the cell. Since neither xylose nor L-sorbose is phosphorylated, in yeast, it is concluded that the increased content of G6P under aerobic conditions inhibits the transport of these non metabolisable sugars (Kleinzeller and Kotyk, 1968).

It can be seen that a substantial amount of evidence suggests that the rate of glucose uptake is regulated by the intracellular content of G6P. This in turn would be expected to depend upon the combined activities of the metabolic pathways utilising that substrate.

PHOSPHOFRUCTOKINASE.

The enzyme isolated from several saccharomyces species, including S.cerevisiae, has been investigated in detail and found to have the same regulatory properties. Therefore no distinction between the particular enzyme source is made here.

PFK is strongly inhibited by ATP (Vinuela, Salas and Sols, 1963; Ramaiah, Hathaway and Atkinson, 1964; Moore, Betz and Chance, 1965; Betz and Moore, 1967) at concentrations found within the living yeast cell (Betz and Moore, 1967). A specific inhibitor site for ATP distinct from the catalytic one exists since other nucleoside triphosphates, which act as substrates, are not inhibitory (Vinuela, Salas and Sols, 1963; Lindell and Stellwagen, 1968). The inhibitory effect of ATP can be reversed by AMP (Moore, Betz and Chance, 1965; Betz and Moore 1967; Lindell and Stellwagen, 1968) whereas ADP does not appear to be an important regulatory factor for yeast PFK (Moore Betz and Chance, 1965; Lindell and Stellwagen, 1968; Betz, 1969).

Both the substrate (F6P) and product (FDP) of PFK reverse competitively the inhibitory effect of ATP (Moore, Betz and Chance, 1965; Betz and Moore, 1967). FDP however is only effective in the presence of low concentrations of F6P and with a high F6P concentration FDP may be slightly inhibitory (Betz and Moore, 1967).

In addition to the previously mentioned effectors of PFK, citric acid has been shown to be an inhibitor and its effect appears to be cooperative with that of ATP (Salas et al, 1965; Sols, 1968). Of the relatively large number of effectors of PFK the most important are ATP, AMP and citric acid (Sols, 1968) whereas F6P and FDP serve to buffer the enzyme against any large changes in the level of its negative modifiers (Betz and Moore, 1967).

Since ATP inhibits and AMP counteracts this inhibitory effect the activity of PFK *in vivo* would be regulated by the energy status of the cell (Ramaiah, Hathaway and Atkinson, 1964).

The presence of two interconvertable forms of PFK has been found in *S.cerevisiae*, comprising an ATP and citric acid insensitive form and a form sensitive to inhibition by ATP and citric acid (Vinuela et al, 1964). PFK is present in both forms in growing yeast whereas in resting cells it occurs mainly in the ATP and citric acid sensitive form. Since the yeast cells used in this present investigation where harvested from the stationary phase and used as resting cell suspensions, it may be assumed that they contained mainly an ATP and citric acid sensitive PFK.

GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE KINASE.

The activity of glyceraldehydephosphate dehydrogenase and phosphoglyceratekinase from S.cerevisiae has not been reported to be effected by factors other than their substrates or products. However, NAD^+, $NADH+H^+$, Pi, ADP and ATP are all involved in the reaction catalysed by these two enzymes and since these factors are important in the overall regulation of glucose metabolism it is for this reason that GAPDH and PGK may be regarded as regulatory enzymes.

PGK is inhibited by high levels of ATP (Hess, 1965b) and subject to control via the ADP supply (Krebs and Kornberg, 1957). Therefore, its activity may be regulated by the energy status of the cell. The activity of GAPDH will be effected by the NAD+ and Pi supply (Krebs and Kornberg, 1957) and since high levels of $NADH+H^+$ are inhibitory to this enzyme (Betz and Chance, 1964) the $NADH+H^+/NAD+$ ratio should be an important factor in regulating the activity of GAPDH.

The $NADH+H^+/NAD$ ratio of aerobic cells is closely related to the ATP/ADP ratio and Pi supply by the effect of these factors upon the rate of oxidation of $NADH+H^+$ via the respiratory chain (Chance, 1955; Chance, 1956; Maitra and Estabrook, 1967). Thus, the activities of GAPDH and PGK may be closely coordinated due to the interrelationship of the $NADH+H^+/NAD+$ and ATP/ADP ratio and therefore may be regarded as a single enzyme system. In addition, GAPDH is strongly inhibited by very low levels of its product 1-3 diphosphoglycerate (Hess, 1965a) so that any change in the activity of PGK, will effect a similar change in the activity of GAPDH.

PYRUVATE KINASE.

The enzyme from S.cerevisiae is activated, allosterically, by FDP (Hess, Haeckel and Brand, 1966). PEP is also an allosteric activator but acts competitively with FDP (Hess, Haeckel and Brand, 1966). The activating effect of both FDP and PEP is counteracted by citric acid and ATP. The effect of ATP however is not specific since other nucleoside triphosphates also inhibit (Hess, Haeckel and Brand, 1966). Since ATP inhibits PK and ADP is a substrate in the reaction it might be expected that, in vivo the enzyme would be sensitive to changes in the energy status of the cell. However, at any one time, the activity of PK should depend upon the combined effect of the relative concentrations of all its effectors ie. FDP, PEP, ATP, ADP and citric acid.

CITRATE SYNTHETASE AND NAD+ ISOCITRATE DEHYDROGENASE.

Citrate synthetase from S.cerevisiae is inhibited by ATP, which decreases the affinity of the enzyme for acetyl CoA (Parvin and Atkinson, 1968) whilst NAD+ isocitrate dehydrogenase is activated by AMP (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963). Thus in vivo both the rate of formation and removal of citric acid would be expected to be regulated by the energy status of the cell.

GLYCOGEN SYNTHETASE.

Both ATP and ADP are allosteric inhibitors of the enzyme from S.cerevisiae and G6P counteracts this inhibition (Rothman and Cabib, 1967). Since both ATP and ADP are inhibitory, whilst AMP has little effect upon the activity of glycogen synthetase, a direct regulation of the activity of this enzyme by the energy status of the cell would not appear to be an important factor in vivo. However, the intracellular content of G6P and therefore the activity of glycogen synthetase is likely to be controlled by the activity of PFK which is sensitive to the energy status of the cell. (Ramaiah, Hathaway and Atkinson, 1964). Therefore it would be expected that in vivo the activity of glycogen synthetase would be closely linked to the energy status of the cell via G6P.

(c). THE CONTROL OF POLYSACCHARIDE SYNTHESIS AND GLUCOSE DEGRADATION IN SACCHAROMYCES CEREVISIA.

C1. GENERAL CONSIDERATIONS.

It can be seen from the properties, discussed in the previous section, of regulatory enzymes concerned with aerobic glucose degradation via the glycolysis system and tricarboxylic acid cycle that they share a common property in that they are sensitive to changes in the level of adenine nucleotides. In general ATP inhibits and either ADP or AMP activates these enzymes. Therefore it might be expected that in vivo the rate of glucose degradation would be regulated by the energy status of the cell.

In addition to an effect of energy status, citric acid would also be expected to be involved in the regulation of aerobic glucose degradation due to the sensitivity of NAD+ isocitrate dehydrogenase to AMP (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963) and

the inhibitory effect of citric acid on PFK (Salas et al, 1965) and PK (Hess, Haeckel and Brand, 1966). Also since PFK is likely to regulate the intracellular content of G6P, which is proposed to regulate the glucose transport system (Sols, 1968) and glycogen synthetase (Rothman and Cabib, 1967) both the rate of glucose uptake and the distribution of incorporated glucose between polysaccharide synthesis and degradation, in aerobic cells, might be expected to be regulated by both the energy status and citric acid content of the yeast cell.

S.cerevisiae contains an active adenylate kinase (Chiu, Su and Russell, 1967) so that any change in ATP will effect an opposite change in ADP and AMP. Therefore a number of terms may be used to represent the energy status of the cell viz. ATP/ADP, ATP/AMP and ATP/ADP+AMP. More recently energy status has been expressed as the energy charge of the adenylate system which is defined as ATP+ $\frac{1}{2}$ADP/ATP+ADP+AMP (Atkinson 1968), a number of enzymes whose activity responds to changes in the level of adenine nucleotides show steep response curves to energy charge in the region of 0.8 and it is suggested that the energy charge of the adenylate system most closely represents the physiological function of the adenylate system in metabolic regulation (Atkinson 1968). However, it still remains true that any change in the level of ATP should result in an opposite change in* here in any interpretation of results, in the literature, where the level of only one of the adenine nucleotides has been reported.

* ADP and AMP and this assumption is used

C2. IN VIVO EVIDENCE.

Resting yeast cells both utilise and degrade glucose at a slower rate under aerobic compared to anaerobic conditions (Pasteur effect). This has been attributed to a lower activity of PFK as indicated by the higher aerobic content of HMP (Lynen et al, 1959; Salas et al, 1965; Kopperschlager, Baehr and Hoffman, 1967). In addition to PFK a lower activity of PGK and PK has been indicated under aerobic compared to anaerobic conditions (Pye, 1965). Lynen et al. (1959) and Salas et al. (1965) found that the content of ATP was essentially the same under both aerobic and anaerobic conditions which lead to the suggestion that the Pasteur effect is regulated by citric acid since this was shown to be markedly higher in aerobic compared to anaerobic cells (Salas et al. 1965; Sols, 1968). However, Kopperschlager, Baehr and Hoffman (1967) found that ATP was higher under aerobic compared to anaerobic conditions whereas the content of citric acid was very similar. Also Pye (1965) found that ADP was lower under aerobic compared to anaerobic conditions. Therefore, it appears that either the citric acid content (Salas et al. 1965; Sols, 1968) or energy status of the cell (Kopperschlager, Baehr and Hoffman, 1967; Pye, 1965) may be the regulatory factor controlling the Pasteur effect in yeast. These conflicting results might be due either to the use of different strains of S.cerevisiae or to differences in the physiological state of the cells used. This might be expected to influence responses to an enviromental change such as the aerobic to anaerobic transition used in studies upon the Pasteur effect (Lynen et al, 1959; Salas et al, 1965; Pye, 1965).

Following the initial studies upon the Pasteur effect most investigations into the regulation of glucose metabolism in yeast

have been concerned with oscillating anaerobic glycolysis. Although changes in the glycolysis flux rate do occur under these conditions they appear to be small and are limited to the rate of accumulation and depletion of intermediates and cofactors within the glycolysis sequence, rather than the overall rate of ethanol and CO_2 production (Betz and Chance, 1964). However it has been proposed that control sites and effectors involved in the generation of oscillations are also concerned with the regulation of nonoscillating and stabilized glycolysis (Hess, 1965b) and therefore should be relevant to the work presented in this thesis.

During oscillatory glycolysis a decreased flux rate is associated with an increase in the energy status (ATP/ADP+AMP) and HMP content of the cells and vice versa (Gosh and Chance, 1964; Betz and Chance, 1964; Hommes, 1964). Thus under these conditions the energy status appears to regulate the glycolysis rate via the effect of ATP and AMP upon the activity of PFK. However, consideration of the phase relationship (Gosh and Chance, 1964) of intermediates and cofactors during the oscillations show that PFK alone cannot be responsible for the generation and maintainence of oscillations (Betz and Chance, 1964). Therefore, regulation of the activities of PFK, PGK and PK by the energy status, together with a close coordination of the activities of GAPDH and PGK, due to the high sensitivity of GAPDH to low levels of its product 1-3 diphosphoglycerate, has been proposed to regulate glycolysis under these conditions (Hess, 1965b). These conclusions are supported by titration studies of oscillating glycolysis in cell extracts, where the characteristics of the oscillations are modified by the addition of intermediates and cofactors (Hess, 1965a; Chance, Schoener and Elsaessar, 1965) and pure enzymes (Hess, 1965a; Hess, Brand and Cassuto, 1965; Hess and Brand, 1965).

The evidence already considered in this section, in relation to the regulation of glucose metabolism, has been concerned solely with the glycolysis system. However, it has been proposed that regulatory changes which effect the rate of glucose degradation via the glycolysis system will also regulate the distribution of incorporated glucose between polysaccharide synthesis and degradation (Ramaiah, Hathaway and Atkinson, 1964; Rothman and Cabib 1967). Until very recently no reports of in vivo studies, with yeast, to support these proposals existed in the literature. Those reports which have recently been published and which support these proposals (Rothman and Cabib 1969; Medrano, Ruiz-Amil and Losada, 1969) will be discussed later in relation to the results presented in this thesis.

Evidence already exists which suggests that glucose metabolism is regulated by the energy status and citric acid content of the yeast cell. However, no detailed investigation has been reported in which both polysaccharide synthesis and glucose degradation has been studied in relation to the intracellular content of intermediates and regulatory compounds. The present investigation was undertaken in an attempt to relate changes in the metabolic fate of incorporated glucose to differences in such factors as the energy status and citric acid content of the yeast cell.

M A T E R I A L S A N D M E T H O D S

ORGANISM. This was a strain of Saccharomyces cerevisiae (baker's yeast), which was obtained by single colony isolation from a block of commercial baker's yeast as supplied by Distillers Co. Ltd., Epsom, England. The yeast was maintained on agar slopes of the medium described by De Kloet, Van Wermeskerken and Koningsberger (1961) and subcultured at weekly intervals.

GLASSWARE. Glassware was washed with ORVUS neutral detergent (Procter and Gamble Ltd., District Sales Office, Bristol, England.) then thoroughly rinsed with tap water and distilled water. Warburg flasks were washed with Decon 75 Concentrate (Medical-Pharmaceutical Developments Ltd., Brighton, England) by boiling in a dilute solution (20 ml concentrate per 1000 ml of distilled water). The flasks were then thoroughly rinsed with tap water and distilled water and dried in a hot air oven. Glassware used for the determination of carbohydrate was soaked overnight in dilute hydrochloric acid then thoroughly rinsed with tap water and distilled water.

CHEMICALS. Unless otherwise stated all chemicals were purchased from British Drug Houses Ltd., Poole, England, and were of analytical reagent grade.

COENZYMES AND SUBSTRATES. Adenosine-5'-triphosphate (crystalline disodium salt), adenosine-5'-diphosphate (trisodium salt), nicotinamide-adenine dinucleotide phosphate (oxidised form, free acid) and nicotinamide adenine dinucleotide (oxidised form, free acid and reduced form, disodium salt) were purchased from Boehringer Corporation (London) Ltd., London, England.
Glycerate-2, 3-diphosphate (crystalline pentacyclohexylammonium salt)

and phosphoenol pyruvate (crystalline tricyclohexylammonium salt) was purchased from Boehringer Corporation (London) Ltd.,

ENZYMES. Glucose-6-phosphate dehydrogenase. D-Glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49. From yeast. 140 U/mg, 140 U/ml. Glucose phosphate isomerase. D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9. From yeast. 350 U/mg, 700 U/ml. Phosphoglucomutase. α-D-Glucose-1, 6-diphosphate: α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1. From rabbit muscle. 100 U/mg, 200 U/ml. 6-Phosphogluconate dehydrogenase. 6-Phospho-D-gluconate: NADP oxidoreductase (decarboxylating) EC 1.1.1.44. From yeast. 12 U/mg, 24 U/ml.

Hexokinase. ATP: D-hexose. 6-phosphotransferase, EC 2.7.1.1. From yeast. 140 U/mg, 1400 U/ml.

Glycerol kinase. ATP: glycerol phosphotransferase, EC 2.7.1.30. From Candida mycoderma. 85 U/mg, 85 U/ml.

Glycerol-3-phosphate dehydrogenase. L-Glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8. From rabbit muscle. 40 U/mg, 400 U/ml.

Triosephosphate isomerase D-Glyceraldehyde-3-phosphate ketol isomerase, EC 5.3.1.1. From Rabbit muscle. 2400 U/mg, 4800 U/ml.

Aldolase. Ketose-1-phosphate aldehyde lyase, EC 4.1.2.7. From rabbit muscle. 90 U/mg, 90 U/ml.

L-Lactate dehydrogenase. L-Lactate: NAD oxidoreductase. EC 1.1.1.27. From rabbit muscle. 360 U/mg, 1800 U/ml.

Pyruvate kinase. ATP: pyruvate phosphotransferase, EC 2.7.1.40 From rabbit muscle. 150 U/mg, 1500 U/ml.

Adenylate kinase. ATP: AMP phosphotransferase, EC 2.7.4.3 From rabbit muscle 360 U/mg, U/ml.

Enolase. 2-Phospho-D-glycerate hydrolyase, EC 4.2.1.11. From rabbit

muscle. 27 U/mg, 270 U/ml.

Phosphoglyceromutase. 2,3-Diphospho-D-glycerate: 2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3. From rabbit muscle. 30 U/mg, 150 U/ml.

L-Malate dehydrogenase. L-Malate: NAD oxidoreductase, EC 1.1.1.37 From pig heart. 720 U/mg, 3600 U/ml.

Citrate Lyase. Citrate oxalacetate-lyase, EC 4.1.36. From Aerobacter aerogenes. 8 U/mg, 40 U/ml.

All the above enzymes were purchase from Boehringer Corporation (London) Ltd., London, England.

UDP glucose dehydrogenase. UDP glucose: NAD oxidoreductase, EC 1.1.1.22 From bovine liver, Type III. 200 U/mg, 100 U/ml. This enzyme was purchased from Sigma (London) Chemical Company Ltd., London, England.

BUFFERS. The buffers used throughout the investigation to suspend yeast cells at the various pH values were:-

(1) 0.04M Orthophosphoric acid - sodium dihydrogen phosphate buffer pH 2.0 and pH 3.0. which was prepared by mixing 0.04M orthophosphoric acid and 0.04M sodium dihydrogen phosphate to give the required pH value.

(2) 0.04M Orthophosphoric acid - disodium hydrogen phosphate buffers pH 4.5, pH 6.5 and pH 7.5 were prepared by mixing 0.04M orthophosphoric acid and 0.04M disodium hydrogen phosphate to give the required pH value.

GROWTH AND PREPARATION OF YEAST. The growth from a slope culture, incubated for 24 hr at 30°, was suspended in sterile distilled water and a standard inoculumn of 25 μg dry wt yeast used to inoculate 75 ml of the liquid medium described by De Kloet et al., (1961).

The cultures were incubated at 30° and 250 rev/min in an orbital shaker (Gallenkamp Ltd., London, England.). Using these conditions the yeast had just entered the stationary phase of growth 18 hr after inoculation of the cultures.

Yeast cells were harvested by centrifuging at 5000 rev/min for 1 min in an M.S.E. 6L refrigerated centrifuge.

Cells were washed once with distilled water and once with buffer, by resuspending the pellet and centrifuging down, using 50 ml washing medium per g wet wt yeast cells. Finally the pellet was resuspended in buffer to the required cell concentration.

DRY WEIGHT DETERMINATION OF THE YEAST. Cellulose acetate filter membranes (Oxoid Ltd., London, England) were dried overnight at 105°, cooled in a desiccator over phosphorous pentoxide and weighed. The membranes were reconstituted by wetting and a known volume of yeast suspension, containing 10-20 mg dry wt, filtered onto the membrane. The membranes and yeast cells were then dried overnight at 105°, cooled in a desiccator and reweighed.

RESPIRATION AND POLYSACCHARIDE SYNTHESIS DETERMINATIONS.

Warburg flasks contained, in the main compartment, yeast cells 10 mg dry wt, sodium phosphate buffer 40 mM and glucose 5 mM in a final volume of 3.0 ml, 0.1 ml 40% (w/v) KoH was included, on filter paper, in the centre well. The gas phase was air. The endogenous respiration was measured for 30 min. when glucose was tipped from the side arm into the main compartment. Flasks were removed at timed intervals, after tipping glucose, and the cells transferred quantitatively onto membrane filters (Oxoid) washed with 2 x 5 ml buffer and transfered to 5 ml 0.5 M trichloroacetic acid (TCA). The extraction was continued overnight at room temperature

when the cell debris and cell extract were made up to 50ml with distilled water. Both total polysaccharide and TCA soluble polysaccharide, (obtained by filtering the suspension through a membrane (Oxoid Ltd) and collecting the filtrate) were estimated by the phenol-sulphuric acid method of Dubois et al., (1956). The initial polysaccharide content of the cells was estimated by treating 2 ml aliquotes of the original cell suspension in an identical manner to that described above.

The filtrate from the cell suspension, at the time of removing the cells from the Warburg flasks, was collected in a test tube and used to determine glucose using a Glucose oxidase test kit (Schweizerhall, Basle, Switzerland) and ethanol by the method of Bonnichsen (1965). Acetaldehyde was determined by the method of Bergemeyer (1965) and acetate by the method of Conway (1962).

At the same time that the respiration rate of cells supplied with exogenous glucose was measured the endogenous respiration rate of cells from the same washed-cell suspension was measured. In experiments to measure the extent of glucose oxidation the oxidation was considered to be complete when the rate with cells receiving glucose had reverted to that of parallel control endogenous cells.

IDENTIFICATION OF THE TRICHLOROACETIC ACID SOLUBLE POLYSACCHARIDE.

The filtrates were adjusted to pH 7.0 with 4N NaOH, freeze dried and deionised by elution through a column of Zeokarb H+ form. (permutit Company Ltd., London, England). and a column of Amberlite IR 4B OH- form (B.D.H. Ltd., Poole, England.). The eluate was freeze dried and partial hydrolysis carried out by refluxing with

IN HCl for 36 hr at 140° and complete hydrolysis by refluxing with 2N H_2SO_4 for 36 hr at 140°. HCl was removed by repeated evaporation to dryness and H_2SO_4 by neutralisation with $Ba(OH)_2$ and removal of the barium sulphate precipitate by centrifugation. A non hydrolysed and the two hydrolysed fractions were chromatographed on thin layer plates of Whatman Microcrystalline Cellulose C.C.41 (H. Reeve Angel and Co Ltd., London, England) using either butan-1-ol-pyridine-water (2:2:1, by vol) or butan-1-ol-ethan-1-ol-water (5:4:1, by vol) as solvents. Components were located on chromatograms with the alkaline silver nitrate spray of Smith (1963).

PREPARATION OF CELL EXTRACTS. A known volume of cell suspension was withdrawn with a pipette and rapidly mixed with perchloric acid to a final concentration of 0.5M $HClO_4$. Extraction was continued for 30 min at room temperature when the extract was adjusted to pH 7.0, on a pH meter, with 40% (w/v) potassium hydroxide. The cell debris and potassium perchlorate were removed by centrifugation and the clear supernatant used to estimate intermediates and adenine nucleotides.

TIME COURSE STUDIES OF INTERMEDIATE AND ADENINE NUCLEOTIDE CONTENT OF YEAST CELLS METABOLLSING GLUCOSE.

Yeast cells harvested and washed as described were suspended, in buffer, to give 1g wet wt yeast per 70 ml buffer. The cell suspensions (in 250ml Erlenmeyer flasks) were equilibrated to 30° by incubating in an orbital shaker for 30 min at 30° and 250 rev/min. Immediately before the addition of glucose to the cells a sample was withdrawn for the preparation of cell extract or determination of polysaccharide. To 70 mls cell suspension 5ml glucose solution,

75mM or 750mM, was added to give initial concentrations of 5mM or 50mM glucose. At time intervals after the addition of glucose samples were withdrawn for the preparation of cell extracts or for determination of polysaccharide. Extracts were used to determine glucose and ethanol as described. Intermediates and adenine nucleotides were determined as described in the following section.

DETERMINATION OF INTERMEDIATES AND ADENINE NUCLEOTIDES.

The procedures used were based on those given in Bergmeyer (1965). Because of the low cell concentration used (13.3 mg wet wt per ml) and the use of 340 mμ absorbance changes to assay the intermediates, relatively large volumes of cell extract were required for the determinations. This effected the rate of the reactions under "standard" conditions. Thus modification of the conditions was necessary and the details are given here. It was determined that the conditions used gave quantitative recovery of commercial preparations of the intermediates when these were added to the extracts.

All determinations were carried out at 30° in a Unicam SP 800 recording spectrometer (Unicam Instruments Ltd., Cambridge, England) fitted with SP 825 programmer controller, SP 830 automatic cell changer, SP 874 constant temperature housing, SP 850 scale expansion accessory and a SP 20 external recorder.

All reactions were continued to completion as monitored by repeated measurements of 340mμ absorbance at 2 min intervals.

D-Glucose-6-phosphate D-Glucose-1-phosphate D-Fructose-6-phosphate and Adenosine-5'-triphosphate. (Hohorst 1965).

Extract		0.500ml
Buffer	(0.1M)	2.000
$MgCl_2$	(0.5M)	0.050
NADP+	(10.0mg/ml)	0.020
Glucose	(0.5M)	0.200
G6PDH*		0.002
PGM		0.002
PGI		0.002
HK		0.005

It was determined in preliminary investigations that, in each condition, the ratio of G6P/F6P was 4.0. Subsequently G6P and F6P were assayed together as hexose monophosphate and the content of the individual hexosemonophosphates calculated from the equilibrium ratio (Hess 1963).

The buffer used in this assay must not be stronger than 0.1M otherwise the hexokinase reaction was inhibited.

*Abbreviations. Glucose-6-phosphate dehydrogenase G6PDH, Phosphoglucomutase PGM, Phosphoglucoseisomerase PGI, Hexokinase HK.

Dihydroxyacetone phosphate, D-Glyceraldehyde-3-phosphate and D-Fructose-1, 6-diphosphate. (Bucher and Hohorst 1965).

Extract		1.000 ml
Buffer	(0.4M)	2.000
$NADH+H^+$	(10.0mg/ml)	0.010
GDH *		0.002
TPI		0.002
ALD		0.002

The content of D-Glyceraldehyde-3-phosphate was too low to be determined accurately, therefore dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate were assayed together as triose phosphates.

*Abbreviations. Glycerol-3-phosphate dehydrogenase GDH, Triosephosphate isomerase TPI, Aldolase ALD.

L-Glycerol-3-phosphate and Glycerol (Wieland 1965)

Extract	0.500 ml
Buffer (0.2M)	2.500
ATP (30mg/ml)	0.100
NAD+ (20mg/ml)	0.050
GDH*	0.010
GK	0.010

The enzymes were inhibited if a larger volume of extract was used.

* Abbreviations. Glycerol-3-phosphate dehydrogenase GDH,
Glycerol kinase GK.

D-3-Phosphoglycerate, D-2-Phosphoglycerate and Phosphoenol pyruvate. (Czok and Eckert 1965).

Extract		1.000ml
Buffer	(0.065M)	1.500
KCl	(4.0M)	0.050
$MgSO_4$	(1.0M)	0.050
ADP	(5mg/ml)	0.050
2,3-di-PG	(8mg/ml)	0.050
$NADH+H^+$	(20mg/ml)	0.020
LDH*		0.002
PK		0.005
ENOL		0.005
PGM		0.005

Use of the low buffer concentration was essential to ensure excess Mg^{2+} in relation to the EDTA content of the buffer.

* Abbreviations. L-Lactate dehydrogenase LDH, Pyruvate kinase PK, Enolase ENOL, Phosphoglycerate mutase PGM, 2,3-diphosphoglycerate 2,3-di-PG.

Pyruvate, Adenosine-5'-diphosphate and Adenosine-5'-monophosphate (Adam 1965).

Extract	1.000 ml
Buffer (0.05M)	2.000
Reaction mixture	0.200
LDH*	0.002
PK	0.005
AK	0.005

The reaction mixture was prepared using twice the recommended concentration of $NADH+H^+$. This was necessary because of the high pyruvate content in extracts prepared from cells suspended at pH 7.5.

The absolute content of AMP could not be determined due to the high AMP content of the $NADH+H^+$. However changes in the AMP content would be expected to be determined during the course of an experiment.

* Abbreviations. L-Lactate dehydrogenase LDH, Pyruvate kinase PK, Adenylate kinase AK.

Oxalacetate and Citrate. (Moellering and Gruber 1966).

Extract		1.000
Buffer	(0.1M)	2.000
$MgCl_2$	(0.5M)	0.010
$NADH+H^+$	(20mg/ml)	0.020
LDH*		0.002
MDH		0.004
CLY		0.020

Magnesium was used in place of zinc because zinc formed a precipitate of zinc phosphate in the cuvettes. The high citrate lyase concentration used ensured excess activity throughout the assay in spite of inactivation of citrate lyase by magnesium.

The buffer must be prepared and stood at room temperature for two days before use. In the presence of freshly prepared buffer a continual decrease in OD340mμ, was obtained when citrate lyase was added to the cuvettes, the reason for which is not understood.

*Abbreviations. L-Lactate dehydrogenase LDH, Malate dehydrogenase MDH and Citrate lyase CLy.

UDP Glucose (Mills and Smith 1965).

Extract		0.500ml
Buffer	(0.1M)	2.000
NAD+	(20 mg/ml)	0.050
UDPGDH*		0.010

The rate of reaction was slow and a larger volume of extract inhibited the reaction completely.

*Abbreviation. Uridine diphospho glucose dehydrogenase UDPG DH.

D-6-Phosphogluconate (Hohorst 1965)

Extract		1.000ml
Buffer	(0.4M)	2.000
$MgCl_2$	(0.5M)	0.050
NADP+	(20mg/ml)	0.010
6-PGDH*		0.005

* Abbreviation. D-6-Phosphogluconate dehydrogenase 6-PGDH

R E S U L T S

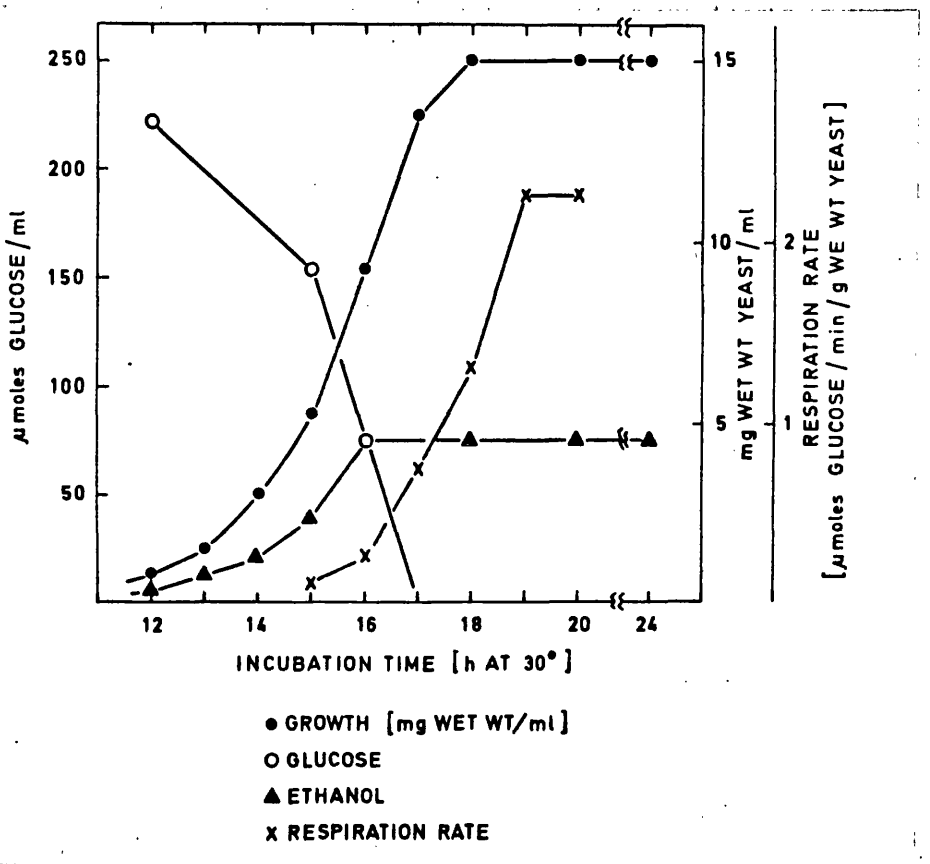
THE GROWTH PATTERN OF YEAST CULTURES AND RESPIRATION OF THE CELLS.

The growth pattern of the yeast cultures is shown in Fig. 1. The cells grew to a final concentration of 5mg dry wt per ml of culture medium and entered the stationary phase 18 hr after inoculation of the medium. Growth ceased soon after glucose became exhausted from the medium and ethanol, formed during the earlier phase of growth was not subsequently utilised. The failure of the yeast to grow on ethanol was not due to depletion of amino acids, vitamins or ammonia. This was determined by doubling the concentration of peptone, yeast extract and ammonium sulphate in the medium both singly and in combination, which did not increase the cell yield even after 48 hr incubation of the cultures.

The respiratory capacity of batch yeast cultures, grown on glucose, increases with increasing age of the culture (Ephrussi et al, 1956; Polakis, Bartley and Meek, 1964). The respiration of yeast cells, harvested at different stages of the growth cycle, was determined using glucose (5mM initial concentration) as the substrate and sodium phosphate buffer (pH 4.5, 40mM) as the suspending medium. The respiratory capacity of the cells increased with increasing age of the culture until a maximum was reached at 19 hr (Fig. 1).

Yeast used throughout this work were harvested at either 18 hr or 20 hr after inoculation of the medium. Yeast cells harvested during the stationary phase would be expected to contain mitochondria (Polakis, Bartley and Meek, 1964; Utter, Duell and Bernofsky, 1968) a competent citric acid cycle and glyoxalate bypass system (Polakis,

FIG 1 AEROBIC GROWTH OF Saccharomyces cerevisiae



Bartley and Meek, 1965) and electron transport system (Polakis, Bartley and Meek, 1964 and 1965; Utter, Duell and Bernofsky, 1968).

1. THE EFFECT OF EXTRACELLULAR pH UPON REGULATION OF GLUCOSE METABOLISM IN 18h CELLS UTILISING 5mM GLUCOSE

1A. THE TOTAL AMOUNT OF GLUCOSE SYNTHESISED TO POLYSACCHARIDE OR DEGRADED VIA RESPIRATION.

The total amount of glucose synthesised to polysaccharide was measured and the total amount degraded via respiration was calculated, at the time when the respiration rate of cells given glucose (375μmoles per g wet wt yeast) returned to that of cells respiring without exogenous substrate. At that time neither glucose, ethanol, acetic acid nor a significant amount of glycerol was present in the extracellular medium, which indicated that, at each pH value, all of the glucose was utilised.

The values obtained are shown in Table 1. from which it can be seen that, more glucose was synthesised to polysaccharide whereas less was degraded,with increasing extracellular pH. The amount of glucose recovered in these two fractions (polysaccharide + respiration) was relatively constant over the pH range and accounted for 60% - 68% of the glucose utilised. Therefore it appeared that the extracellular pH did not influence the total amount of glucose which was potentially available to either the polysaccharide synthesising or glucose degrading systems, but that it caused a changed distribution of a fixed proportion of incorporated glucose between these systems.

TABLE 1. THE TOTAL AMOUNT OF GLUCOSE SYNTHESISED TO POLYSACCHARIDE OR RESPIRED BY 18h CELLS (5mm GLUCOSE)

μ moles GLUCOSE EQUIVALENT PER g. WET WT. YEAST	pH		
	2.0	4.5	7.5
Polysaccharide. n = 10	28 ± 5	86 ± 5	118 ± 7
Respiration. n = 10	226 ± 5	143 ± 4	106 ± 3
Polysaccharide + Respiration	254	229	224
% Glucose recovered (375 = 100%)	68	61	60

1B. THE AMOUNT OF GLUCOSE TAKEN UP AND ETHANOL PRODUCED AT 10 MIN.

The amount of glucose taken up and degraded, during the first 10 min of glucose utilisation, was measured to determine whether the changed distribution of glucose between polysaccharide synthesis and degradation (Table 1) might have been due to an effect upon the glucose transport system (Van Steveninck and Dawson, 1968). If this was the case then it would be expected that any effect of extracellular pH upon glucose uptake would be reflected by a similar effect upon glucose degradation (Van Steveninck, 1966). It was found that ethanol was formed at each pH value and since no significant amount of oxygen was consumed during the first 10 min of glucose utilisation, glucose degradation could be simply expressed as the amount of ethanol formed.

The results obtained are shown in Table 2. At both pH 2.0 and pH 7.5 glucose uptake was reduced to a very similar extent compared to pH 4.5. However, the amount of ethanol produced at pH 2.0 was twice that at pH 7.5. In addition the proportion of the glucose taken up which was degraded to ethanol, decreased with increasing extracellular pH. Therefore there was not a simple relationship between the effect of extracellular pH upon glucose uptake and degradation. In some manner the activity of the glycolysis system appeared to be altered so that glycolysis was favoured at acid pH.

The difference in the amount of glucose taken up and ethanol formed at pH 2.0 compared to pH 4.5, and at pH 7.5 compared to pH 4.5 is also shown in table 2. At pH 7.5 both glucose uptake and ethanol production were reduced to a similar extent compared to pH 4.5. This indicated that the same process might have controlled the activity

TABLE 2. THE AMOUNT OF GLUCOSE TAKEN UP AND ETHANOL PRODUCED AT 10 MIN BY 18h CELLS (5mm GLUCOSE).

μmoles GLUCOSE EQUIVALENT PER g WET WT YEAST	pH		
	2.0	4.5	7.5
Glucose taken up n = 10	122 ± 9	201 ± 12	131 ± 8
Ethanol produced n = 10	80 ± 5	95 ± 8	32 ± 4
Ethanol as % Glucose	66	47	25
Difference Glucose taken up from pH 4.5	79	-	70
Difference Ethanol produced from pH 4.5	15	-	63

of both the glucose transport and glycolysis system at pH 7.5 and also, from the reverse comparison, at pH 4.5. At pH 2.0 however, ethanol production was reduced to a much lesser extent than glucose uptake compared to pH 4.5. This indicated that the same process did not control the activity of both the glucose transport and glycolysis system at pH 2.0.

1C. THE INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE FRUCTOSE DIPHOSPHATE CITRIC ACID AND ADENINE NUCLEOTIDES AT 10 MIN.

1. HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE.

The intracellular content of HMP (G6P + F6P) and FDP was measured at 10 min after addition of glucose to the cells to determine:

(a) Whether phosphofructokinase controlled the activity of the glycolysis system at either pH value, by comparing any differences in the intracellular content of HMP with those in the amount of ethanol formed, between the three pH values (Newsholme and Gevers, 1967).

(b) Whether the activity of the glucose transport system was controlled by extracellular pH (Van Steveninck and Dawson, 1968) or by feed back regulation via HMP (Sols, 1968) by comparing differences between the three pH values in the intracellular content of HMP and the amount of glucose taken up.

The intracellular content of HMP and FDP at 10 min after addition of glucose to the cells is shown in Table 3. The intracellular content of HMP increased with increasing extracellular pH whereas that of FDP was highest at pH 2.0. Therefore, since both HMP and FDP were not changed in the same manner it was possible to determine whether differences in the intracellular content of HMP were due to differences in the activity of phosphofructokinase (Newsholme and Gevers, 1967). This is most conveniently done by comparing the pH 7.5 with the pH 4.5 condition and the pH 2.0 with both the pH 7.5 and pH 4.5 conditions.

pH 7.5 A higher intracellular content of HMP (Table 3) at pH 7.5 was associated with a lower ethanol production (Table 2) compared to pH 4.5. Therefore, the activity of phosphofructokinase was lower at pH 7.5 than at pH 4.5 (Newsholme and Gevers, 1967). In addition, the higher intracellular content of HMP at pH 7.5 was associated with a lower glucose uptake (Table 2) compared to pH 4.5. This may indicate that at pH 7.5 and by the reverse comparison at pH 4.5, the activity of the glucose transport system was regulated by the intracellular content of HMP (Sols, 1968) and not by extracellular pH (Van Steveninck and Dawson, 1968). Further since both glucose uptake and ethanol production were changed by a similar amount between the pH 4.5 and pH 7.5 conditions (Table 2) it could be concluded that at these two pH values, phosphofructokinase controlled the activity of both the glycolysis system and, via HMP, the glucose transport system.

pH 2.0 A lower intracellular content of HMP (Table 3) at pH 2.0 was associated with a higher ethanol production (Table 2) compared to pH 7.5. Using the previous criterion this means that the activity of phosphofructokinase was higher at pH 2.0 than at pH 7.5 (Newsholme and Gevers, 1967). However, the higher activity of phosphofructokinase at pH 2.0 did not result in a higher glucose uptake (Table 2) compared to pH 7.5, which indicated that at pH 2.0 the activity of the glucose transport system was reduced by a direct effect of extracellular pH (Van Steveninck and Dawson, 1968). This is further indicated by the fact that at pH 2.0 a reduced intracellular content of HMP was associated with a reduced glucose uptake (Table 2) compared to pH 4.5.

In summary a comparison of differences in the intracellular content of HMP with those of glucose uptake and ethanol production indicated

TABLE 3. THE INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE FRUCTOSE DIPHOSPHATE CITRIC ACID AND ADENINE NUCLEOTIDES AT 10 MIN IN 18h CELLS (5mM GLUCOSE)

μmoles PER g WET WT YEAST.	pH		
	2.0	4.5	7.5
Hexose Monophosphate (G6P+F6P) n = 11	1.30 ± 0.05	2.05 ± 0.06	2.90 ± 0.15
Fructose Diphosphate n = 11	1.60 ± 0.07	0.40 ± 0.01	0.36 ± 0.01
Citric Acid n = 6	1.13 ± 0.17	1.22 ± 0.04	1.17 ± 0.10
Adenosine Tri-phosphate n = 11	1.82 ± 0.01	2.10 ± 0.20	1.70 ± 0.14
Adenosine Diphosphate n = 9	0.90 ± 0.07	0.42 ± 0.08	0.27 ± 0.01
Adenosine Mono-phosphate n = 7	0.15 ± 0.02	< 0.05	< 0.05
Energy Status (ATP/ ADP)	2.0	5.0	6.3

the following. The activity of phosphofructokinase may have decreased with increasing extracellular pH. Furthermore at pH 4.5 and pH 7.5 the activity of phosphofructokinase controlled the activity of both the glycolysis and glucose transport systems. At pH 2.0 however an additional effect of extracellular pH upon the activity of the glucose transport system limited the supply of substrate to phosphofructokinase. The high activity of this enzyme at pH 2.0 was expressed only as a higher proportion of glucose being degraded during the first 10 min of glucose uptake (Table 2).

2. CITRIC ACID AND ADENINE NUCLEOTIDES

The intracellular content of both citric acid (Salas et al, 1965) and adenine nucleotides (Vinuela, Salas and Sols, 1963; Ramaiah, Hathaway and Atkinson, 1964; Betz and Moore, 1967) was measured at 10 min after addition of glucose to the cells to determine whether the observed differences in the activity of phosphofructokinase (see 1Ci, p54) might have been due to differences in the intracellular content of these reported effectors.

The results obtained are shown in Table 3. At pH 2.0 the intracellular content of ATP was lower and that of ADP and AMP higher than at both pH 4.5 and pH 7.5. In addition the energy status of the cells, expressed as ATP/ADP, increased with increasing extracellular pH. Therefore the low level of ATP and high level of AMP (Vinuela, Salas and Sols, 1963; Betz and Moore, 1967) and low energy status (Atkinson, 1968) could have caused the high activity of phosphofructokinase at pH 2.0. In addition the higher energy status (Atkinson, 1968) at pH 7.5 could have been responsible for the lower activity of phosphofructokinase in cells suspended at that extracellular pH value compared to pH 4.5.

Clearly the differences in the activity of phosphofructokinase, at 10 min, could not have been due to citric acid (Salas et al, 1965) since its intracellular content was the same at all three pH values.

SUMMARY. The results obtained with 18h cells could be interpreted in the following manner:- In some way changes in extracellular pH affected the energy status of the cells. This increased with increasing extracellular pH (Table 3). This change in turn altered the activity of phosphofructokinase which decreased with increasing extracellular pH (see 1Ci, p54) which resulted in a lower proportion of the incorporated glucose being degraded with increasing extracellular pH (Table 2). If these relationships observed at 10 min were maintained throughout the course of glucose uptake then less of the available glucose would be degraded and therefore more synthesised to polysaccharide with increasing extracellular pH: cf changes in extracellular pH effected a changed distribution of a fixed proportion of the glucose utilised (Table 1). The fact that less glucose was degraded via respiration with increasing extracellular pH would be explained by oxidation of ethanol formed during glucose uptake (Eaton and Klein, 1957) cf the proportion of glucose degraded to ethanol decreased with increasing extracellular pH (Table 2) but ethanol was absent from the extracellular medium when respiration was judged to be complete.

2. THE EFFECT OF EXTRACELLULAR pH UPON REGULATION OF GLUCOSE METABOLISM IN 20h CELLS UTILISING 5mM GLUCOSE

The results obtained with 18h cells suggested that phosphofructokinase might regulate the distribution of incorporated glucose between the polysaccharide synthesising and glycolysis systems (Ramaiah, Hathaway and Atkinson, 1964). To determine if this was in fact the case, it was necessary to measure rates of glucose uptake, glycolysis and polysaccharide synthesis and compare any differences, between the three extracellular pH values, with differences in the intracellular content of glycolysis intermediates and effectors of phosphofructokinase during the same time periods.

Experiments of this type were begun using cells harvested at 18h after inoculation of the cultures. However, at this stage of the investigations certain difficulties in growth of the cultures occurred. Thus cells harvested from some 18h cultures exhibited a very low rate of glucose uptake at pH 2.0. An investigation of the effect of cell age upon glucose uptake at pH 2.0 revealed that the rate of glucose uptake, at this pH value, increased with increasing cell age and became optimal when the cells had just entered the stationary phase of the growth cycle ie at 18h in the "normal" growth curve shown in Fig 1. Therefore the low rate of glucose uptake by some 18h cells could be explained by the cells being physiologically younger than the majority of cells harvested at that time.

In order to bypass this problem it was decided to use cells harvested at 20h after inoculation of the cultures. These older cells never exhibited an abnormally low rate of glucose uptake at pH 2.0. Therefore after establishing that extracellular pH exerted a similar effect to that observed with 18h cells, upon the amount of glucose synthesised to polysaccharide or degraded via respiration by 20h cells, this work was continued using 20h cells.

2A. THE TOTAL AMOUNT OF GLUCOSE SYNTHESISED TO POLYSACCHARIDE OR DEGRADED VIA RESPIRATION.

The effect of extracellular pH upon the total amount of glucose synthesised to polysaccharide or degraded via respiration by 20h cells is shown in Table 4. More glucose was synthesised to polysaccharide whereas less was degraded with increasing extracellular pH. The differences in these parameters was not due to a reduced glucose utilisation at either pH since neither glucose, ethanol, acetic acid nor a significant amount of glycerol was present in the extracellular medium at the time when polysaccharide synthesis and respiration of exogenous substrate was judged to be complete.

The amount of glucose recovered in the two fractions (polysaccharide + respiration) was similar at the three pH values and accounted for 65% - 77% of the glucose utilised. Therefore it could be concluded that extracellular pH caused a changed distribution in a fixed proportion of incorporated glucose.

TABLE 4. THE TOTAL AMOUNT OF GLUCOSE SYNTHESISED TO POLYSACCHARIDE OR RESPIRED BY 20h CELLS (5mM GLUCOSE).

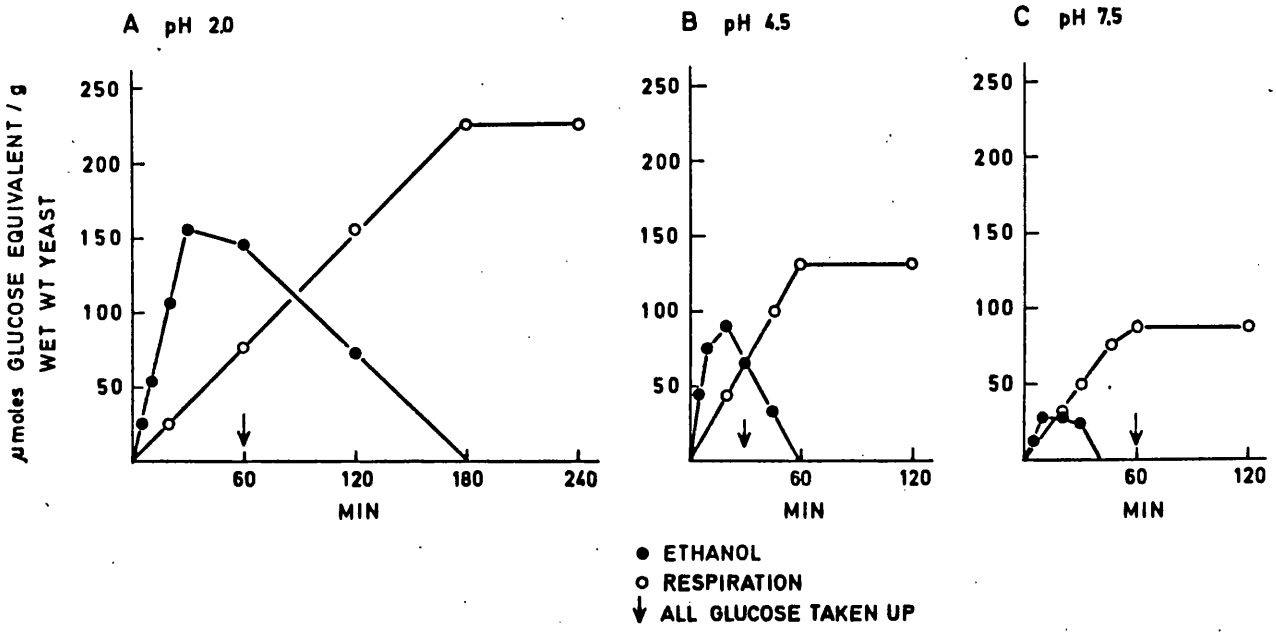
μmoles GLUCOSE EQUIVALENT PER g WET WT YEAST	pH		
	2.0	4.5	7.5
Polysaccharide. n = 6	63 ± 5	110 ± 7	157 ± 14
Respiration. n = 3	225 ± 5	130 ± 6	88 ± 10
Polysaccharide + Respiration	288	240	245
% Glucose recovered (375 = 100%)	77	64	65

2B. ETHANOL PRODUCTION AND UTILISATION.

In the following section the amount of glucose degraded, at any one time, is expressed as the sum of ethanol present in the extracellular medium plus the amount of oxygen consumed by the cells. In order to show that glucose was initially degraded via the glycolysis system the time course of ethanol production is presented here. The time course of subsequent ethanol utilisation and of respiration is also shown to demonstrate the metabolic fate of ethanol formed during glucose uptake. It may be assumed that ethanol was not produced to any marked extent by the combined operation of the glycolysis and oxidative pentose phosphate pathways, since this latter pathway operates to a very low extent in resting cells of Saccharomyces cerevisiae and may account for as little as 3% of the glucose utilised under very similar conditions to those used in this work (K. Brand, personnel communication 1970).

It can be seen from Fig.2 that the amount of ethanol formed decreased with increasing extracellular pH and therefore was influenced in the same manner as the amount of glucose respired (see Table 4). Ethanol formed during glucose utilisation was subsequently utilised and was in fact oxidised completely. This is shown by the fact that at pH 2.0 and pH 4.5, when all of the glucose had been utilised (30 min pH 4.5, 60 min pH 2.0) the rates of ethanol uptake and respiration were very similar. In addition the rate of respiration, at these two pH values decreased to the endogenous level when all of the ethanol had been taken up. At pH 7.5 respiration continued above the endogenous rate after all the ethanol had been taken up but returned to the endogenous rate when all of the glucose had been utilised (60 min). Presumably at this pH value the rates of glycolysis and respiration were more closely co-ordinated between 40-60 min.

FIG 2 ETHANOL PRODUCTION ETHANOL UTILISATION AND RESPIRATION (5 mM GLUCOSE)



for mean values ± SEM see APPENDIX 1.

Thus the amount of glucose degraded to ethanol, via glycolysis, decreased with increasing extracellular pH and, since it was subsequently oxidised completely, the amount of glucose respired also decreased with increasing extracellular pH.

2C. THE RATE OF GLUCOSE UPTAKE, GLYCOLYSIS AND POLYSACCHARIDE SYNTHESIS.

The rate of glucose uptake, glycolysis and polysaccharide synthesis at the three pH values, was measured and compared to determine whether the changed distribution of glucose between polysaccharide synthesis and glycolysis might be due to a direct effect of extracellular pH upon the glucose transport system. If this was the case then it could be expected that there would be a simple relationship between any differences in the rates of the three pH values (Van Steveninck, 1966). Here glycolysis is expressed as the sum of ethanol formed plus oxygen consumed at any one time. That this may be used to express the activity of the glycolysis system, under these aerobic conditions, was shown in the previous section 2B, p64).

GLUCOSE UPTAKE FIG 3A. The rate of glucose uptake was highest at pH 4.5 and was reduced to the same extent at pH 2.0 and pH 7.5. Therefore at these latter two pH values the activity of the glucose transport system was reduced compared to pH 4.5.

GLYCOLYSIS. FIG 3B. During the first 10 min, when a constant rate was maintained at each pH value the rate of glycolysis was highest at pH 4.5 as was the case with glucose uptake (Fig. 3A). However, at pH 2.0, the rate of glycolysis was higher than at pH 7.5 which did not reflect the effect of extracellular pH upon the rate of glucose uptake, which was the same at pH 2.0 and pH 7.5 (Fig 3A).

After 10 min the rate of glycolysis decreased markedly at both pH 4.5 and pH 7.5 but at pH 2.0 was maintained constant until 30 min. This resulted in more glucose being degraded at pH 2.0 than at pH 4.5.

POLYSACCHARIDE SYNTHESIS FIG 3C.

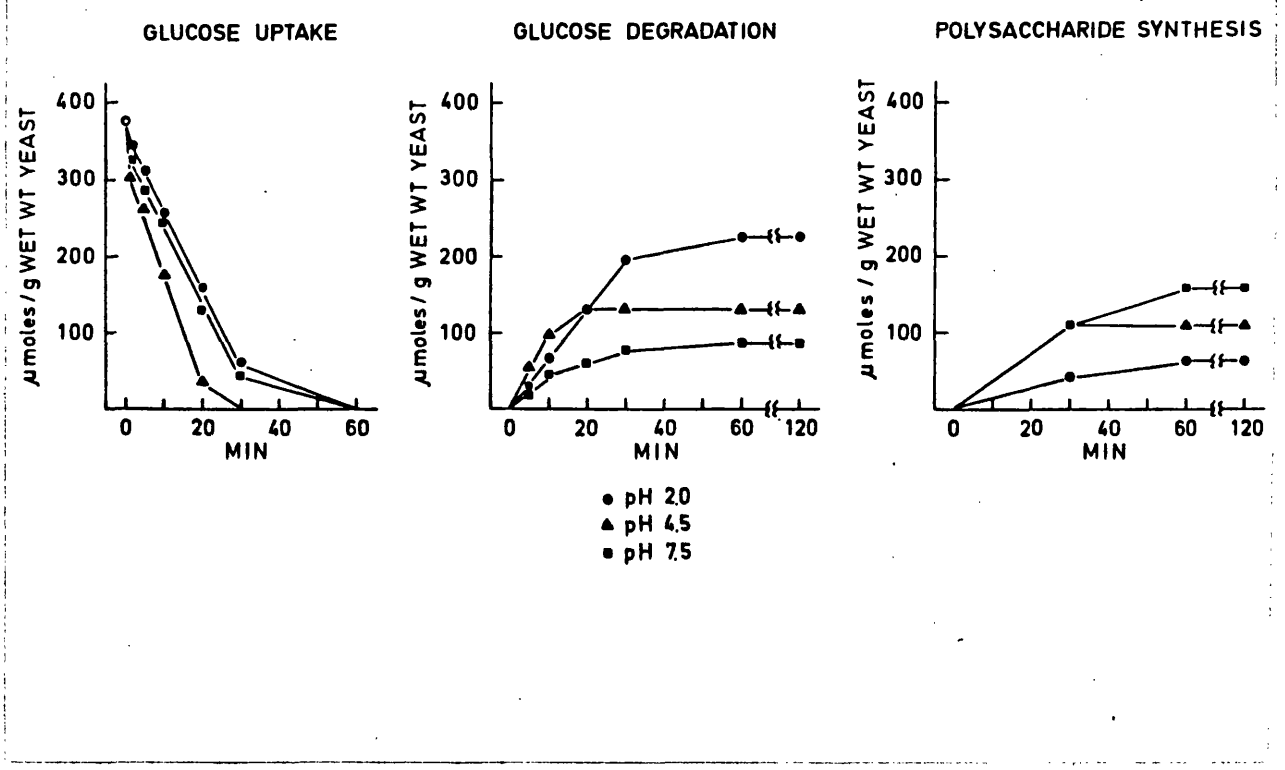
The rates of polysaccharide synthesis were the same at pH 4.5 and pH 7.5 and the rate at these two pH values were higher than at pH 2.0. Therefore there was no simple relationship between the rates of polysaccharide synthesis and glucose uptake since a) the same rate of polysaccharide synthesis was achieved at pH 4.5 and pH 7.5 with different rates of glucose uptake b) different rates of polysaccharide synthesis were achieved at pH 2.0 and pH 7.5 with the same rate of glucose uptake.

Although the rate of polysaccharide synthesis was the same at pH 4.5 and pH 7.5 more polysaccharide was eventually synthesised from the available glucose at pH 7.5. This could be related to the slower rate of glucose uptake at pH 7.5 (Fig 3A) which allowed polysaccharide synthesis to continue for a longer time than at pH 4.5.

It should be noted that polysaccharide synthesis ceased, at each pH value, at the same time that all of the glucose had been taken up (30 min pH 4.5, 60 min pH 2.0 and pH 7.5. cf Fig 3A and Fig 3C). Also the maximum amount of glucose had been degraded (ethanol + respiration) at the same time that all of the glucose had been taken up (cf FIG 3A and Fig 3B). Therefore the changed distribution of incorporated glucose, caused by changes in extracellular pH (Table 4 and Fig 3B and Fig 3C) occurred only during glucose uptake. This indicated that the relative activities of the polysaccharide synthesising and glycolysis systems were effected in some manner by extracellular pH.

A comparison of the amount of glucose taken up and degraded at 10 min between the pH 2.0 and pH 4.5 conditions and the pH 7.5 and pH 4.5

FIG 3 GLUCOSE UPTAKE GLUCOSE DEGRADATION AND POLYSACCHARIDE SYNTHESIS (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM: GLUCOSE UPTAKE APP 2
GLUCOSE DEGRADATION APP 1
POLYSACCHARIDE SYNTHESIS APP 3

TABLE 5. THE AMOUNT OF GLUCOSE TAKEN UP AND DEGRADED AT 10 MIN BY 20h CELLS (5mM GLUCOSE).

μmoles GLUCOSE EQUIVALENT PER g WET WT YEAST.	pH		
	2.0	4.5	7.5
Glucose taken up n = 4	110 ± 10	199 ± 4	131 ± 15
Glucose Degraded n = 4	67 ± 6	97 ± 4	44 ± 3
Difference Glucose taken up from pH 4.5	89	-	68
Difference Glucose degraded from pH 4.5	30	-	53

conditions is shown in Table 5. At pH 7.5 both glucose uptake and glycolysis was reduced to a similar extent compared to pH 4.5. This indicated that at both pH 4.5 and pH 7.5 the activity of the glucose transport and glycolysis systems were controlled by the same process. At pH 2.0 however the activity of the glycolysis and glucose transport system could not have been controlled by the same process, since glycolysis was reduced to a lesser extent than glucose uptake compared to pH 4.5.

2D. THE INTRACELLULAR CONTENT OF GLYCOLYSIS INTERMEDIATES, CITRIC ACID AND ADENINE NUCLEOTIDES DURING GLUCOSE UTILISATION.

A comparison of the rates of glucose uptake, polysaccharide synthesis and glycolysis at the three pH values indicated that the relative activities of the polysaccharide synthesising and glycolysis systems might have been effected by changes in extracellular pH (see 2C, p67). To determine how the activity of the glycolysis system might have been effected the intracellular content of glycolysis intermediates, citric acid and adenine nucleotides was measured throughout the course of glucose uptake at the three pH values. The results obtained are presented in the following order:-

1. Hexose monophosphate (G6P + F6P) and fructose diphosphate. These are considered in relation to the activity of phosphofructokinase and the glucose transport system.
2. Citric acid and adenine nucleotides. These are considered in relation to the activity of phosphofructokinase.
3. Hexose monophosphate (G6P + F6P), fructose diphosphate, triose phosphates (DHAP + GAP), phosphoglyceric acids (3PGA + 2PGA), phosphoenolpyruvate and pyruvic acid. These are considered in relation to the activity of pyruvate kinase.

2D1. HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE.

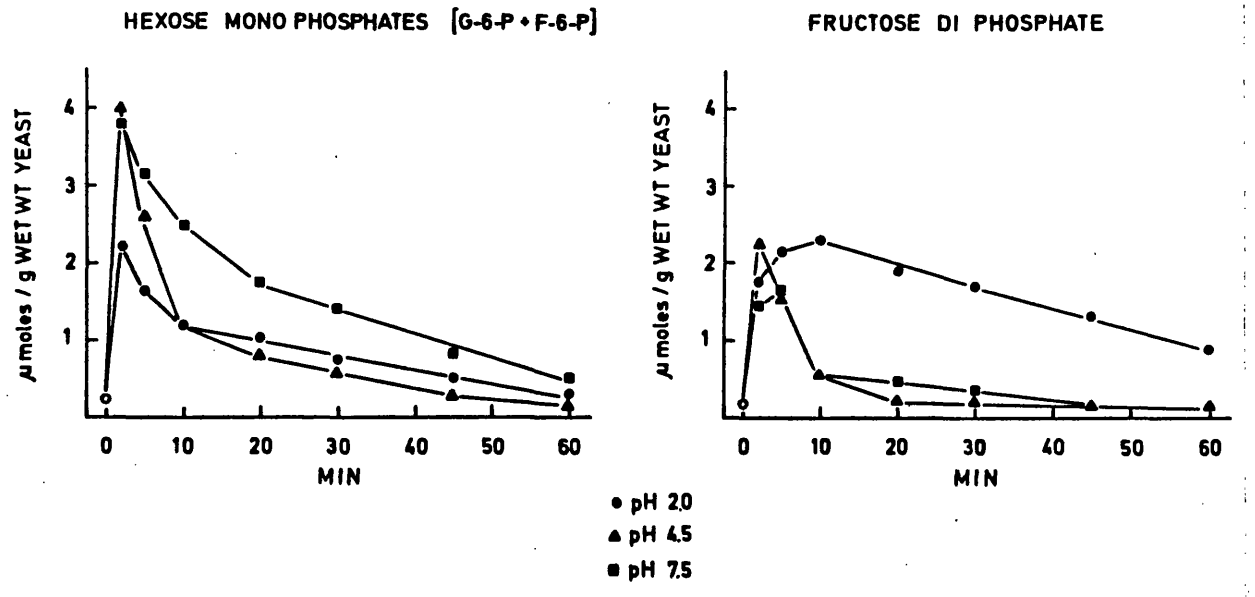
FIG.4.

From Fig 4 it can be seen that there were differences in the intracellular content of HMP (G6P + F6P) (Fig.4A) and FDP (Fig.4B) between the three pH values and that both were not changed in a similar manner. Therefore differences in the intracellular content of HMP could be correlated with those in the rates of both glycolysis and glucose uptake in order to determine whether they might have been controlled by the activity of phosphofructokinase (Newsholme and Gevers, 1967). This is most conveniently done by comparing the pH 7.5 with the pH 4.5 condition and the pH 2.0 with both the pH 7.5 and pH 4.5 conditions.

pH 7.5. A higher intracellular content of HMP (Fig 4A) at pH 7.5 was associated with a lower rate of both glycolysis (Fig 3B) and glucose uptake (Fig 3A) compared to pH 4.5. Therefore, it appeared that at pH 7.5 a low activity of phosphofructokinase, indicated by the high intracellular content of HMP and low rate of glycolysis at this pH value (Newsholme and Gevers, 1967), reduced the activity of both the glycolysis and glucose transport system (Sols, 1968). By making the reverse comparison it can be concluded that the high rate of glycolysis and glucose uptake at pH 4.5 (Fig 3) was due to a high activity of phosphofructokinase. These conclusions are supported by the fact that at 10 min both the amount of glucose taken up and the amount degraded via glycolysis was changed by a very similar extent between the pH 4.5 and pH 7.5 conditions (Table 5).

pH 2.0 A lower intracellular content of HMP (Fig 4A) was associated with a higher rate of glycolysis (Fig 3B) compared to pH 7.5. Therefore the activity of phosphofructokinase was higher at pH 2.0 than pH 7.5 (Newsholme and Gevers, 1967). However this higher

FIG 4 INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM: pH 2.0 APP 4.
pH 4.5 APP 5.
pH 7.5 APP 6.

activity of phosphofructokinase and lower intracellular content of HMP, at pH 2.0, did not result in a higher rate of glucose uptake compared to pH 7.5 (Fig 3A). This suggested that, at pH 2.0, the activity of the glucose transport system was reduced by a direct effect of extracellular pH (Van Steveninck and Dawson, 1968). This is clearly shown to be the case by the fact that, at pH 2.0, a lower intracellular content of HMP (Fig 4A) during the first 10 min of glucose uptake, was associated with a lower rate of glucose uptake (Fig 3A) compared to pH 4.5.

Thus is appeared that at pH 2.0 a high activity of phosphofructo-kinase existed but that the rate of glucose uptake was limited by a direct effect of extracellular pH upon the activity of the glucose transport system. This conclusion is supported by the fact that at pH 2.0 the amount of glucose taken up was reduced to a greater extent than the amount degraded via glycolysis, compared to the pH 4.5 condition (Table 5).

FACTORS CONTROLLING CHANGES IN THE INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE AT ONE pH VALUE.

It can be seen from Fig 4A that differences in the intracellular content of HMP, and therefore the activity of phosphofructokinase, were maintained throughout the course of glucose uptake at pH 4.5 compared to pH 7.5 and at pH 2.0 compared to pH 7.5. However the intracellular content of HMP at pH 4.5 was very similar to that at pH 2.0 after 10 min. This low intracellular content of HMP at pH 4.5 appeared to be due to substrate limitation of the glycolysis system which is indicated by the fact that the rate of glycolysis did not increase during the first 10 min and actually decreased after 10 min, at pH 4.5 (Fig 3B). Therefore the low intracellular content could not be due to an increased rate of removal.

If this was in fact the case it should be possible to determine this by comparing the changes in both HMP and FDP during glucose uptake. Thus a very similar decrease in the intracellular content of both HMP and FDP, when the rate of glycolysis does not increase, would indicate substrate limitation of the glycolysis system, rather than changes in enzyme activity, to be the cause of the decrease. The same criteria can also be applied to the pH 2.0 and pH 7.5 condition to determine why changes in the intracellular content of HMP and FDP occurred at these two pH values.

In Fig 5 the percentage change in HMP and FDP (calculated from the 2 min values) at the three pH values is shown. At pH 4.5 (Fig 5B) both HMP and FDP decreased to a very similar extent after 2 min. Therefore the low intracellular content of HMP, after 10 min, may be attributed to substrate limitation of the glycolysis system. (The fact that substrate limitation occurred in the pH 4.5 condition does

not invalidate the conclusion that the activity of phosphofructokinase was different at this pH value to the pH 2.0 and pH 7.5 condition).

This could be shown by using 50mM in place of 5mM glucose. Thus with this higher glucose concentration, with which a steady state level of intermediates was achieved after 10 min, changes in the intracellular content of HMP and FDP, during the first 10 min, followed the same pattern as those with 5mM glucose (Fig 6A). The initial rise and subsequent fall of the intracellular content of HMP and FDP was a normal overshoot process before these intermediates adjusted to their steady state levels. However with 5mM glucose substrate limitation occurred just before the 10 min point (see lower HMP and FDP at 10 min with 5mM glucose, Fig 6A) so that HMP and FDP did not equilibrate to their steady state levels but continued to decrease.

In Fig 6B changes which occurred in the intracellular content of HMP and FDP at the three pH values during the first 10 min are shown. It can be seen that at any single pH value the extent and characteristics of the overshoot process was essentially the same with both 5mM and 50mM glucose and also that between the three pH values it was markedly different. Thus the activity of phosphofructokinase controlled both the extent and characteristics of changes in the intracellular content of HMP and FDP during the first 10 min of glucose utilisation, and therefore conclusions based upon differences in intermediates and flux rates during that time are in fact valid.

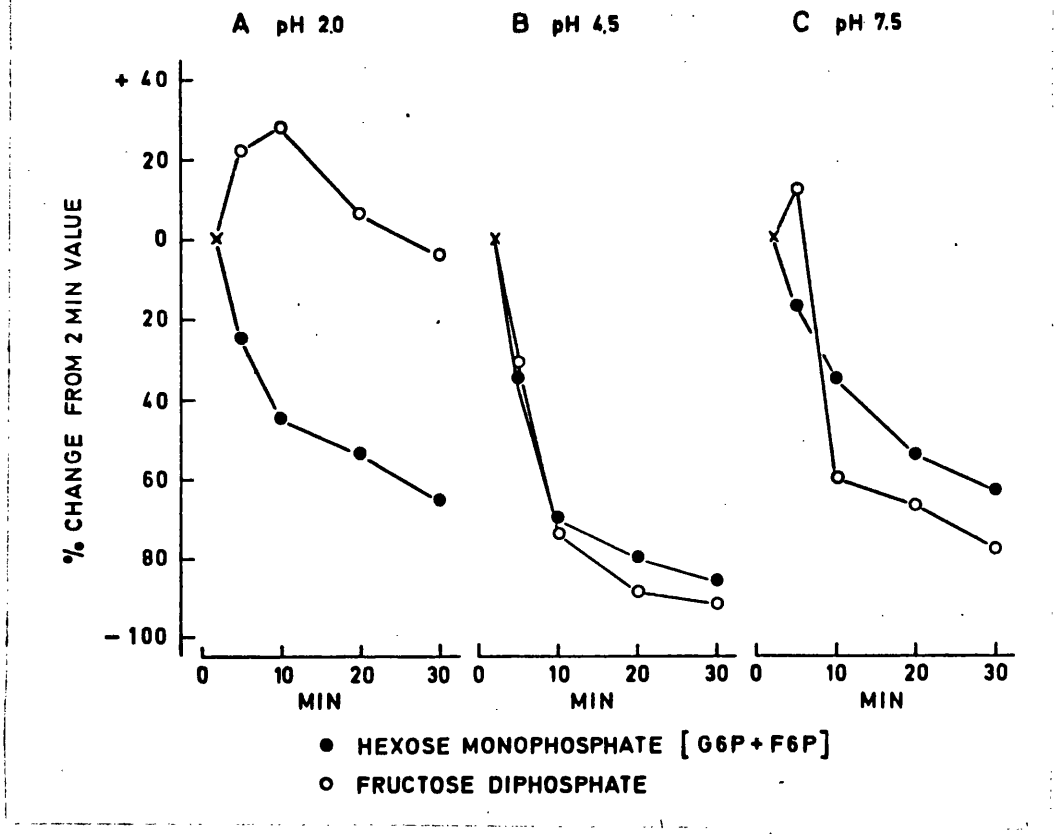
In Fig 5C the percentage change of HMP and FDP in the pH 7.5 condition (5mM glucose) is shown. At this pH value, between 5-10 min, the intracellular content of FDP decreased to a greater extent than that of HMP. This was not due to an increased rate of removal since the rate

of glycolysis did not increase during the first 10 min and actually decreased after 10 min (Fig 3B). Therefore the larger decrease in FDP than HMP, at pH 7.5, may be attributed to a decrease in the activity of phosphofructokinase between 5-10 min. A similar decrease, between 5-10 min, in the activity of phosphofructokinase occurred in cells given 50mM glucose (cf similar changes in HMP and FDP, 5-10 min, with both 5mM and 50mM glucose at pH 7.5, Fig 6B), after which these intermediates equilibrated to a true steady state level with 50mM glucose (not shown here but see Fig 12). However a true steady state level was not achieved after 10 min with 5mM glucose (see slow decrease HMP after 10 min at pH 7.5, Fig 4A). Therefore substrate limitation did occur to a certain degree with 5mM glucose in cells suspended at pH 7.5 but to a much lesser extent than at pH 4.5. This was presumably due to the lower activity of phosphofructokinase and therefore lower rate of glycolysis and glucose uptake at pH 7.5 compared to pH 4.5.

At pH 2.0 (5mM glucose, Fig 5A) the intracellular content of FDP continued to increase until 10 min whereas that of HMP decreased. This together with high intracellular content of FDP (Fig 4B) low intracellular content of HMP (Fig 4A) and high rate of glycolysis (Fig 3B) maintained at pH 2.0 showed that a high activity of phosphofructokinase was maintained throughout the time that glucose was being taken up at this pH value.

Thus at pH 2.0 a high rate of glycolysis was maintained, after 10 min, due to the maintainence of a high activity of phosphofructokinase. At pH 4.5 the rate of glycolysis decreased after 10 min due to substrate limitation of the glycolysis system, whereas at pH 7.5 the rate of glycolysis decreased, after 10 min, mainly due to a decrease in the activity of phosphofructokinase.

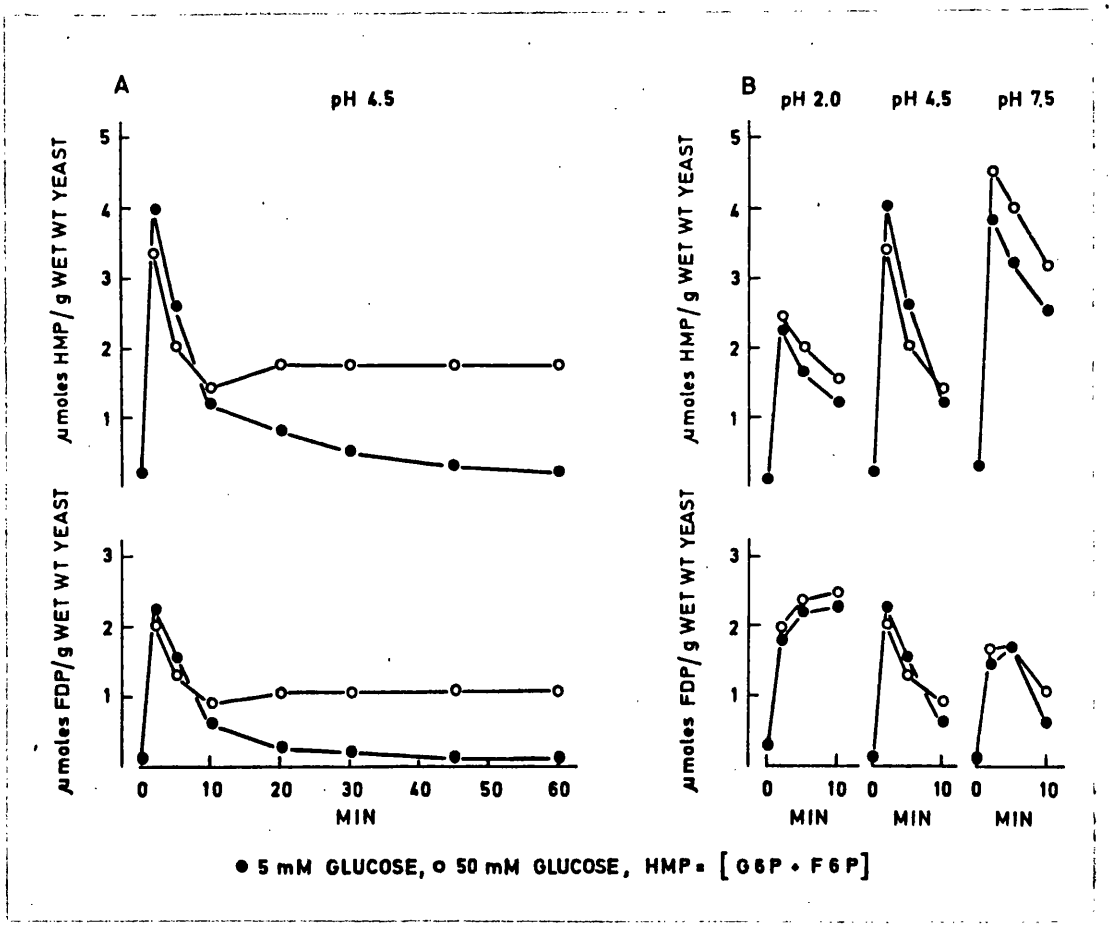
FIG 5 PERCENT CHANGE FROM 2 min VALUE OF HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM of HMP and F6P

pH 2.0 APP 4.
pH 4.5 APP 5.
pH 7.5 APP 6.

FIG 6 THE INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE WITH 5mM & 50 mM GLUCOSE



SUMMARY.

1. In some manner changes in extracellular pH effected the activity of phosphofructokinase which appeared to decrease with increasing extracellular pH.

2. At pH 2.0 an additional effect of extracellular pH upon the glucose transport system limited the rate of glucose uptake and therefore the rate of glycolysis and polysaccharide synthesis. The rate of polysaccharide synthesis was further reduced, at pH 2.0, due to a high activity of phosphofructokinase which caused a high proportion of the incorporated glucose to be degraded at any one time. Since a high activity of phosphofructokinase was maintained throughout the time that glucose was being taken up the total amount of polysaccharide synthesised was reduced and the total amount degraded was increased compared to pH 4.5 and pH 7.5.

3. At pH 4.5 and pH 7.5 the relative activities of the polysaccharide synthesising and glycolysis systems was altered. This was due to a lower activity of phosphofructokinase at pH 7.5 which decreased the activity of the glycolysis system whilst that of the polysaccharide synthesising system remained the same (Fig 3C). The lower activity of phosphofructokinase at pH 7.5 was maintained throughout the time that glucose was being taken up so that less glucose was degraded compared to pH 4.5. In addition since the low activity of phosphofructokinase also reduced, via HMP, the rate of glucose uptake, polysaccharide synthesis continued for a longer time so that more was eventually synthesised from the available glucose at pH 7.5 compared to pH 4.5.

2Dii. CITRIC ACID AND ADENINE NUCLEOTIDES. FIG 7. FIG 8

The intracellular content of ATP and ADP at the three pH values and the change which occurred in the intracellular content of AMP at pH 2.0 is shown in Fig 7. It can be seen that, during glucose utilisation the intracellular content of ATP increased (except 2 min point) and that of ADP decreased with decreasing extracellular pH. In addition, at pH 2.0, the intracellular content of ATP decreased whereas that of AMP increased during glucose utilisation.

Thus differences in the intracellular content of adenine nucleotides, between the three pH values, were consistent with the observed differences in the activity of phosphofructokinase since:

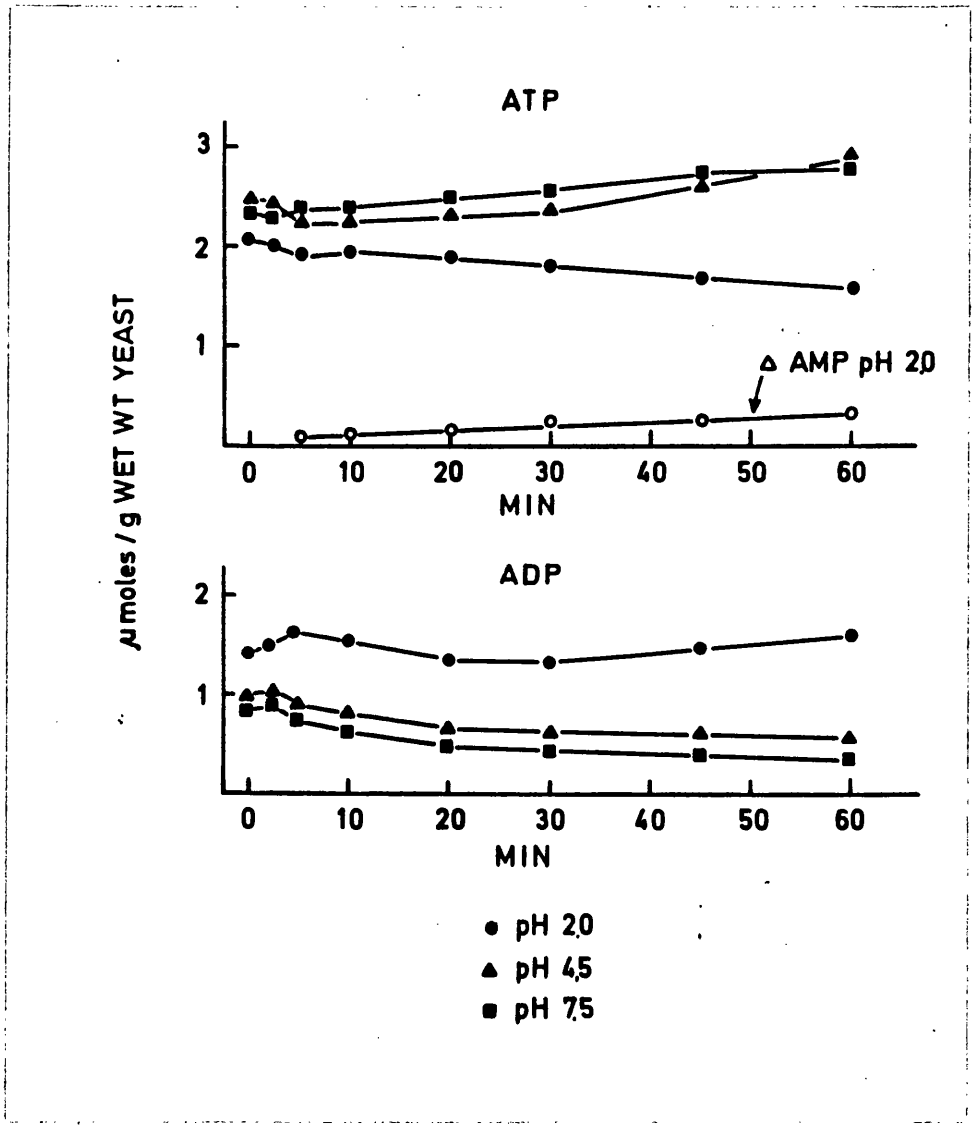
i. The increasing intracellular content of ATP, with increasing extracellular pH, would be expected to result in a decreasing activity of phosphofructokinase (Vinuela, Salas and Sols, 1963; Betz and Moore, 1967) which was in fact observed.

ii. The decreasing intracellular content of ATP and increasing intracellular content of AMP, at pH 2.0, would be expected to maintain a high activity of phosphofructokinase (Ramaiah, Hathaway and Atkinson, 1964; Betz and Moore, 1967) which was in fact observed.

In Fig 8, the energy status, expressed as ATP/ADP, and intracellular content of citric acid is shown. Both the energy status (Atkinson, 1968) and citric acid content (Salas et al, 1965) of the cells, increased with increasing extracellular pH and therefore would be expected to result in the observed differences in the activity of phosphofructokinase.

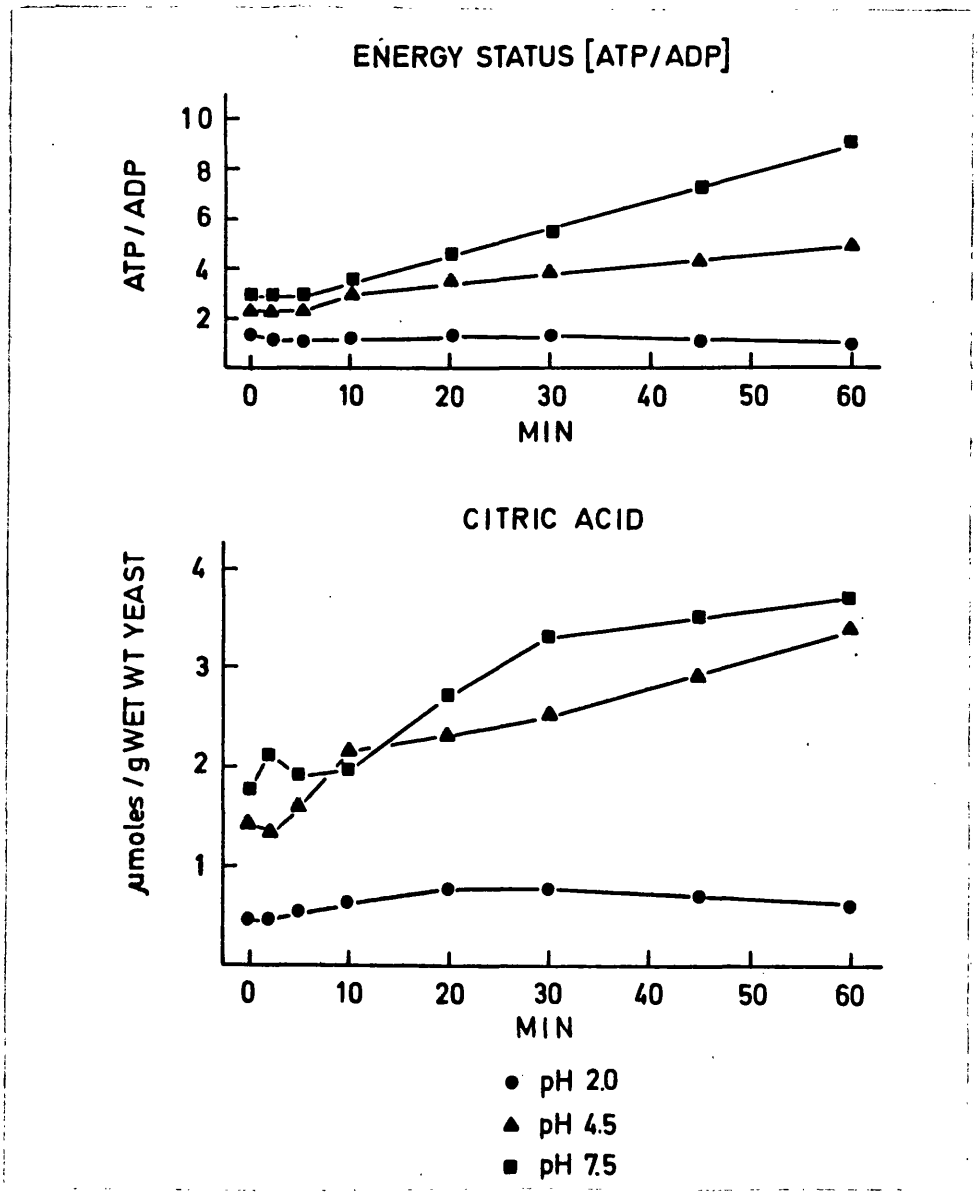
Thus it appeared that the differences in the activity of phosphofructokinase between the three pH values (see 2D1, p72) were consistent with an effect of extracellular pH upon the energy status and citric acid content of the cells.

FIG 7 INTRACELLULAR CONTENT OF ADENINE NUCLEOTIDES (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM: pH 2.0 APP 7.
pH 4.5 APP 8.
pH 7.5 APP 9.

FIG 8 ENERGY STATUS AND INTRACELLULAR CONTENT OF CITRIC ACID (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM:
ATP, ADP pH 2.0 APP 7. pH 4.5 APP 8. pH 7.5 APP 9.
CITRIC ACID APP 10.

2Diii. HEXOSE MONOPHOSPHATE FRUCTOSE DIPHOSPHATE TRIOSE PHOSPHATE PHOSPHOGLYCERIC ACID PHOSPHOENOLPYRUVATE AND PYRUVIC ACID (REGULATION OF PYRUVATE KINASE).

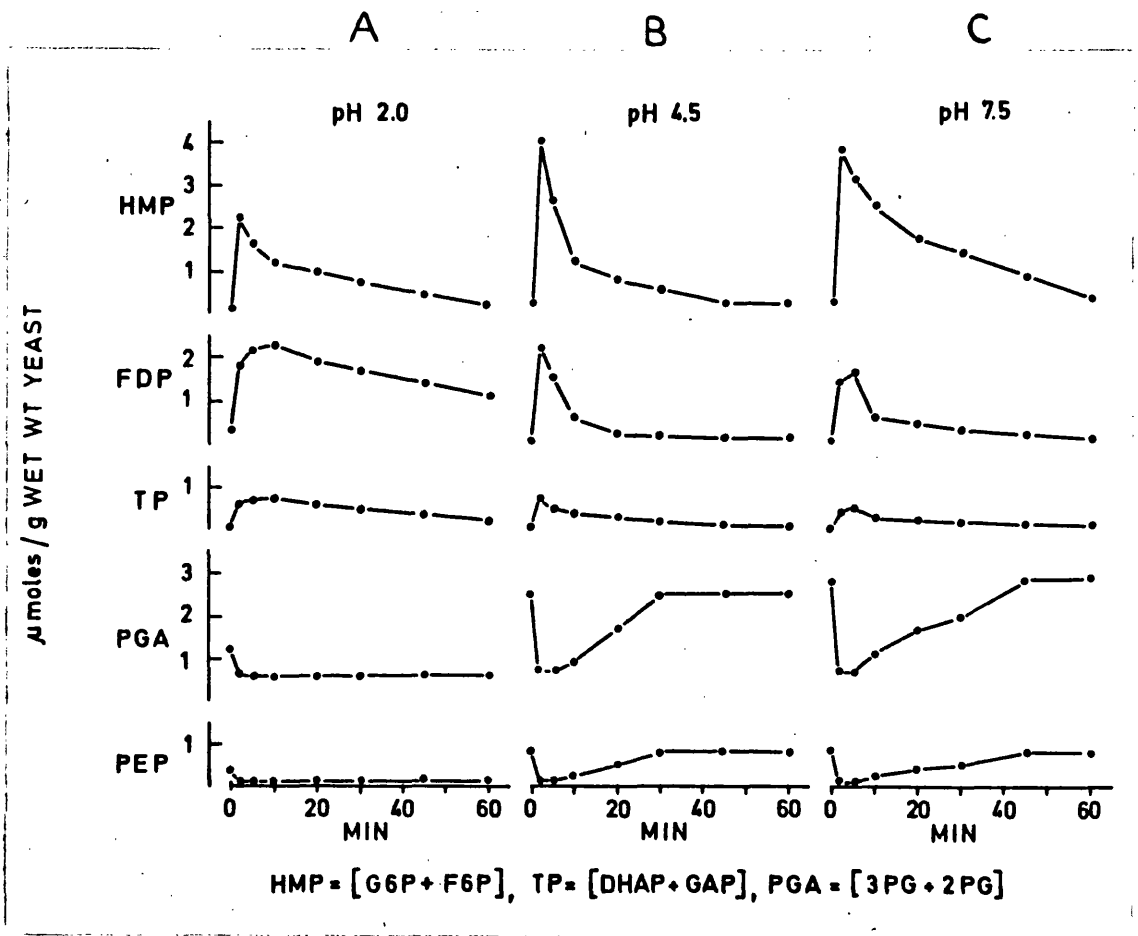
At each pH value no change in the intracellular content of pyruvic acid was detected during glucose utilisation compared to the resting condition. This fact is important in relation to interpretation of changes in the activity of pyruvate kinase.

The changes which occurred in the intracellular content of HMP (G6P + F6P), FDP, TP (DHAP + GAP) PGA (3PGA + 2PGA) and PEP, during glucose utilisation at the three pH values are shown in Fig 9A-pH 2.0, Fig 9B- pH 4.5 and Fig 9C - pH 7.5.

At each pH value, when glucose was added to the cell suspensions (0 min), the intracellular content of HMP, FDP and TP increased whereas that of PGA and PEP decreased. This decrease in the intracellular content of PGA and PEP was associated with an increased glycolytic flux of transition from the resting to the active state of the glycolysis system (see Fig 3B). Therefore during the transition from the resting to the active metabolic state, the lower glycolysis sequence containing the enzymes phosphoglycerate mutase, enolase and pyruvate kinase was activated by some factor other than substrate supply (Newsholme and Gevers, 1967).

After 10 min, at pH 4.5 and pH 7.5 the intracellular content of HMP, FDP and TP was decreasing whilst that of PGA and PEP was increasing (see Fig 9B and Fig 9C) and the intracellular content of intermediates returned to the resting condition when glucose uptake and glycolysis had ceased (30 min pH 4.5, 60 min pH 7.5 - see Fig 3A and Fig 3B). The increasing intracellular content of PGA and PEP was associated with a decreasing glycolytic flux (see decrease in rate of glycolysis

FIG 9 INTRACELLULAR CONTENT OF GLYCOLYSIS INTERMEDIATES (5 mM GLUCOSE)



see APPENDIX for mean values ± SEM: pH 2.0 APP 4.
pH 4.5 APP 5.
pH 7.5 APP 6.

after 10 min, Fig 3B). Therefore during that time the activity of the lower glycolysis sequence was decreasing and as with the initial activation must have been due to some factor other than substrate supply (Newsholme and Gevers, 1967).

At pH 2.0 (Fig9A) unlike the pH 4.5 and pH 7.5 conditions, the lower glycolysis sequence remained activated throughout the course of glucose uptake. This is indicated by the low intracellular content of PGA and PEP (Fig 9A) and the high rate of glycolytic flux (Fig 3B) maintained at this pH value.

The changes which occurred in the intracellular content of PGA and PEP in these cells may be attributed to changes in the activity of pyruvate kinase since

i. The intracellular content of pyruvic acid did not change in a similar manner to that of PEP

ii. The reaction catalysed by pyruvate kinase (PEP + ADP $\longrightarrow$ PYRUVATE + ATP) is essentially irreversible (Krebs and Kornberg,1957). Therefore any large changes in the intracellular content of PEP, which are inversely related to changes in the rate of glycolytic flux, must be due to changes in the activity of pyruvate kinase (Newsholme and Gevers, 1967).

This being the case it would be expected that a change in the intracellular content of PEP would effect, by mass action, a similar change in that of 2PGA and 3PGA due to the freely reversible nature of the reactions catalysed by enolase and phosphoglycerate mutase (Krebs and Kornberg, 1957). Evidently mass action effects would not be expected to alter the intracellular content of intermediates of those reactions catalysed by enzymes above phosphoglycerate mutase, in the glycolysis sequence, due to the difficulties involved in

reversing the reactions catalysed by phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase (Krebs and Kornberg, 1957).

Thus it appeared that pyruvate kinase controlled the activity of the lower glycolysis sequence in these cells, and that its activity was regulated by some factor other than substrate supply.

The activity of pyruvate kinase from S.cerevisiae is regulated in vitro by a number of effectors. These are FDP which is a very strong activator, ADP which also activates and ATP and citric acid which inhibit the enzyme competitively with FDP and ADP (Hess, Haeckel and Brand, 1966; Haeckel et al, 1968). Thus it should be possible to determine whether any of these effectors might have regulated the activity of pyruvate kinase in these cells, by correlating changes in their intracellular content with changes in the activity of pyruvate kinase.

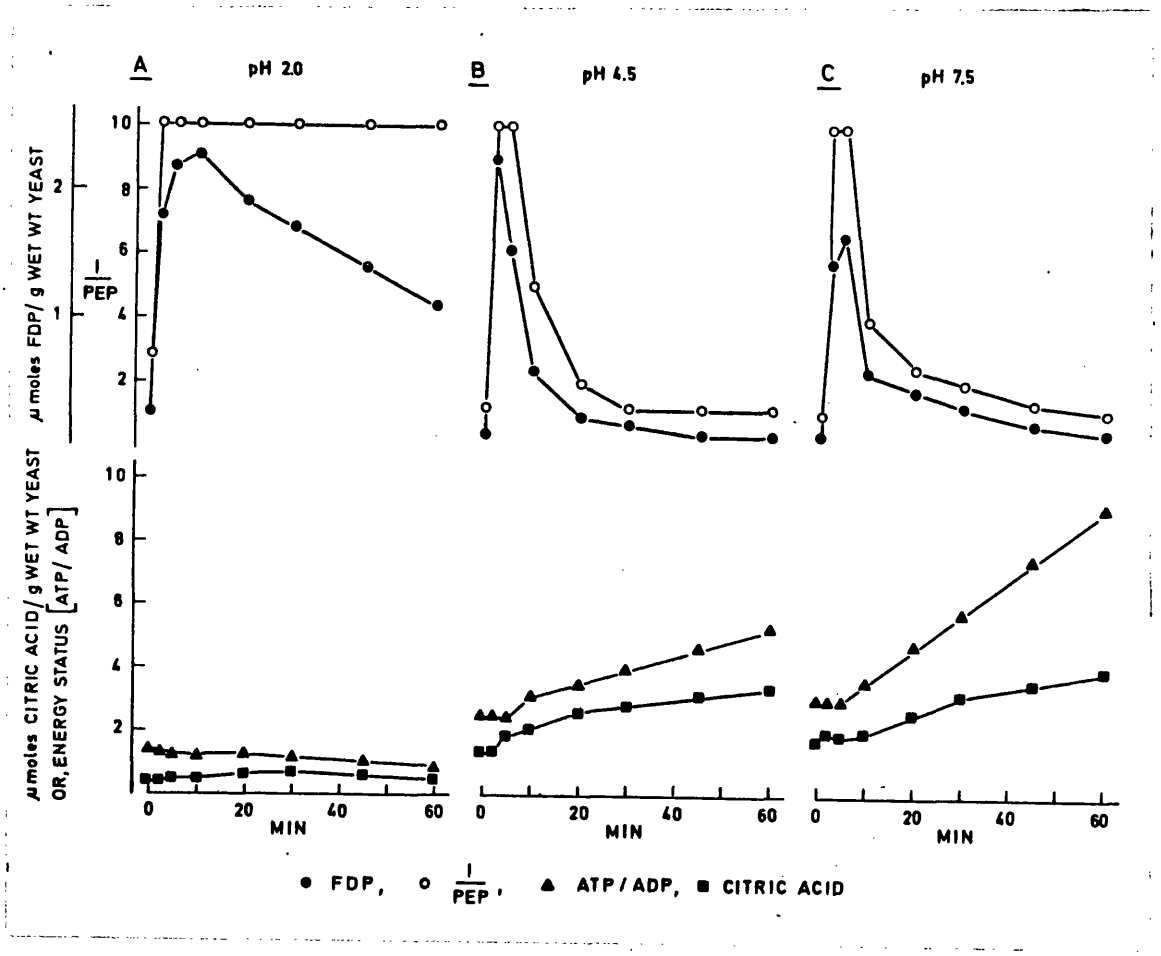
NB. Changes in the activity of pyruvate kinase, in these cells, were inversely related to changes in the intracellular content of PEP. Therefore changes in the activity of pyruvate kinase could be represented by changes in the factor $\frac{1}{PEP}$. Thus an increase in the activity of pyruvate kinase would be represented by an increase, and a decrease in its activity by a decrease in the factor $\frac{1}{PEP}$. This procedure is used here.

CORRELATION WITH FRUCTOSE DIPHOSPHATE. FIG 10.

At each pH value the initial activation of pyruvate kinase was associated with an increase in the intracellular content of FDP. At both pH 4.5 (Fig 10B) and pH 7.5 (Fig 10C) the activity of pyruvate kinase began to decrease when FDP had fallen to a low level (0.6 μ moles per g wet wt yeast at 10 min) and thereafter a decreasing

intracellular content of FDP was associated with a decreasing activity of phosphofructokinase. At pH 2.0 (Fig 10A) where a high activity of pyruvate kinase was maintained throughout the course of glucose uptake a relatively high intracellular content of FDP was also maintained. Therefore there was a direct relationship between changes in the intracellular content of FDP and changes in the activity of pyruvate kinase.

FIG 10 THE RELATIONSHIP BETWEEN CHANGES IN THE ACTIVITY OF PYRUVATE KINASE THE INTRACELLULAR CONTENT OF FRUCTOSE DIPHOSPHATE AND CITRIC ACID AND ATP/ADP RATIO (5mM GLUCOSE)



see APPENDIX for mean values ± SEM:
FDP, PEP pH 2.0 APP 4. pH4.5 APP 5. pH 7.5 APP 6.
ATP, ADP pH 2.0 APP 7. pH4.5 APP 8. pH 7.5 APP 9.
CITRIC ACID APP 10.

CORRELATION WITH ADENINE NUCLEOTIDES AND CITRIC ACID. FIG 10.

Since pyruvate kinase from S.cerevisiae is inhibited by ATP and activated by ADP (Haeckel et al, 1968) the ratio of these two effectors should be the most important factor, in respect to the adenine nucleotides, in regulating the activity of pyruvate kinase. Therefore, changes in the activity of pyruvate kinase were correlated with changes in the ATP/ADP ratio, as well as those of citric acid.

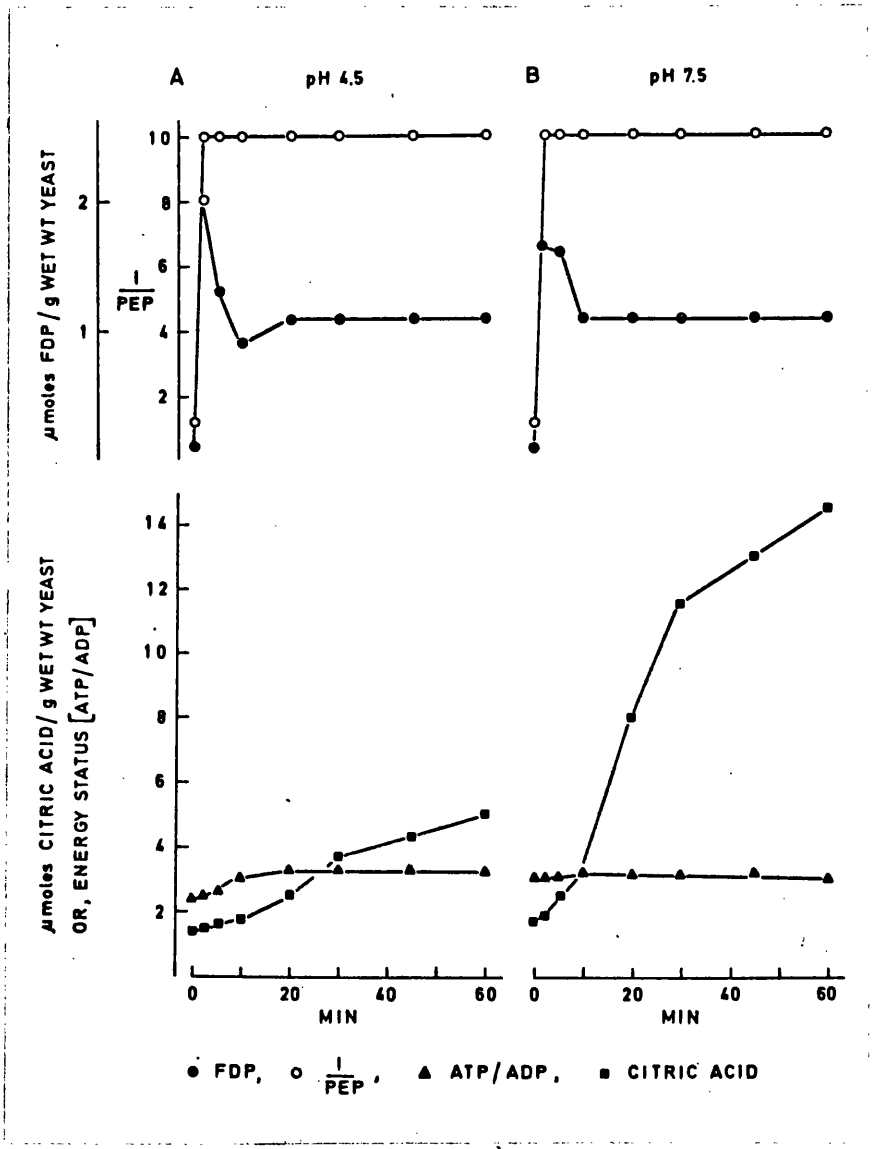
At 2 min the ATP/ADP ratio was the same and the intracellular content of citric acid was not lower compared to the resting condition (0 min) at all three pH values. Therefore, the initial activation of pyruvate kinase (see 0-2 min, Fig 10) could not be attributed to either ATP, ADP or citric acid. However, an increasing ATP/ADP ratio and intracellular content of citric acid at pH 4.5 (Fig 10B) and pH 7.5 (Fig 10C) may have contributed to the decreasing activity of pyruvate kinase, apparent after 10 min, at these two pH values. Also the maintainence of a low ATP/ADP ratio and intracellular content of citric acid at pH 2.0 (Fig 10A) might have contributed to the maintainence of a high activity of pyruvate kinase at this pH value.

Additional evidence to support the role of FDP in the regulation of pyruvate kinase in these cells, was obtained by using 50mM in place of 5mM glucose, at pH 4.5 and pH 7.5. Thus when 50mM glucose was added to the resting cell suspensions pyruvate kinase was activated (Fig 11) as with 5mM glucose (Fig 10). However unlike the 5mM condition, the activity of pyruvate kinase did not decrease after 10 min, in cells utilising 50mM glucose, and a high activity was maintained throughout the time of the experiment (60 min). as was both a high rate of glucose uptake and glycolysis. (Not shown here but values contained in Table 7.

Fig 11 also shows that the initial activation of pyruvate kinase, at both pH 4.5 (Fig 11A) and pH 7.5 (Fig 11B), was associated with an increase in the intracellular content of FDP, whilst neither the ATP/ADP ratio nor the intracellular content of citric acid decreased. Also the maintainence of a high activity of pyruvate kinase was associated with a steady state level of FDP (1.0μmoles per g wet wt yeast) which was higher than the level at which the activity of pyruvate kinase began to decrease in cells given 5mM glucose (0.6μmoles per g wet wt yeast, see 10 min Fig 10B and Fig 10C).

The high activity of pyruvate kinase was maintained at pH 7.5 in spite of the intracellular content of citric acid increasing to 14.5μmoles per g wet wt yeast (see 60 min point Fig 11B). This clearly indicated that citric acid was not a strong effector of pyruvate kinase in the presence of a high intracellular content of FDP.

FIG 11 THE RELATIONSHIP BETWEEN CHANGES IN THE ACTIVITY OF PYRUVATE KINASE THE INTRACELLULAR CONTENT OF FRUCTOSE DIPHOSPHATE AND CITRIC ACID AND ATP/ADP RATIO (50 mM GLUCOSE)



Summary. In the resting condition the activity of pyruvate kinase was considerably reduced and may have been under the control of the adenine nucleotides and citric acid. When glucose was added to the cells the activity of pyruvate kinase was increased due to an increase in the intracellular content of FDP and the activity of this enzyme began to decrease if the intracellular content of FDP fell below about 0.6μmoles per g wet wt yeast.

The intracellular content of FDP could be controlled by substrate supply of the pH 4.5 condition with 5mM or 50mM glucose or via the activity of phosphofructokinase of the pH 2.0 and pH 7.5 conditions with 5mM glucose. Therefore the activating effect of FDP upon pyruvate kinase ensured a high rate of its own removal under conditions where a high rate of formation existed. In addition it is clear that the enzyme phosphofructokinase played an important role in coordinating, via FDP, the activities of the upper and lower glycolysis sequence in the cells used here. These *in vivo* results therefore supported the proposed feed forward role of FDP in the regulation of glycolysis in *S.cerevisiae*, based upon *in vitro* studies. (Hess, Haeckel and Brand, 1966; Haeckel et al, 1968).

2E. THE INTRACELLULAR CONTENT OF GLYCOLYSIS INTERMEDIATES CITRIC ACID AND ADENINE NUCLEOTIDES IN THE RESTING CONDITION.

In those experiments where the intracellular content of intermediates and adenine nucleotides was measured during glucose utilisation, the cell suspensions were equilibrated to 30° by incubating them at 250 rev/min for 30 min in a rotary shaker. Then immediately before addition of glucose to the cell suspensions an aliquot was withdrawn for the determination of the resting level (0 min point) of intermediates and adenine nucleotides.

The values obtained are shown in Table 6. It can be seen that the intracellular content of HMP increased with increasing extracellular pH, whereas that of FDP was highest at pH 2.0. Also that the intracellular content of PGA and PEP was highest at pH 2.0 and that both the energy status (ATP/ADP) and citric acid content of the cells increased with increasing extracellular pH.

The only available measure of glycolytic flux in the resting condition, in this work, was the rate of endogenous respiration, which was unaffected by changes in extracellular pH during the 30 min equilibration period. However this does not necessarily mean that differences in the rate of glycolytic flux may not have existed in the resting condition, because the endogenous substrate for respiration and the pattern of endogenous metabolism is not known for the cells used here.

TABLE 6. THE INTRACELLULAR CONTENT OF GLYCOLYSIS INTERMEDIATES CITRIC ACID AND ADENINE NUCLEOTIDES IN THE RESTING CONDITION - 20h CELLS.

μmoles PER g WET WT YEAST	pH		
	2.0	4.5	7.5
Hexose Monophosphate n = 4 (G6P + F6P)	0.14 ± 0.10	0.26 ± 0.07	0.30 ± 0.01
Fructose Diphosphate n = 4	0.31 ± 0.10	0.09 ± 0.10	0.11 ± 0.05
Triose Phosphate n = 4 (DHAP + GAP)	0.19 ± 0.04	0.10 ± 0.05	0.10 ± 0.05
Phosphoglyceric Acid n = 2 (3PGA + 2PGA)	1.25	2.50	2.78
Phosphoenolpyruvate n = 2	0.35	0.80	0.88
Pyruvic Acid n = 4	0.10 ± 0.01	0.12 ± 0.01	0.10 ± 0.05
Citric Acid n = 4	0.45 ± 0.15	1.40 ± 0.14	1.73 ± 0.14
Adenosine Triphosphate n = 4	2.10 ± 0.10	2.50 ± 0.10	2.35 ± 0.10
Adenosine Diphosphate n = 4	1.40 ± 0.28	1.00 ± 0.17	0.75 ± 0.14
Energy Status (ATP/ADP)	1.5	2.5	3.1

With the above reservation in mind the differences between the pH values in the resting condition suggest the following interpretation. Changes in extracellular pH effected both the energy status and citric acid content of the cells, which in turn altered the activity of phosphofructokinase (Atkinson, 1968; Salas et al 1965). which appeared to decrease with increasing extracellular pH. In addition, at pH 2.0 the lower ATP/ADP ratio and citric acid content, and higher intracellular content of FDP increased the activity of pyruvate kinase compared with pH 4.5 and pH 7.5 (Hess, Haeckel and Brand, 1966; Haeckel et al, 1968).

Thus differences between the three pH values in the resting condition were qualitatively very similar to those found during glucose utilisation. This would suggest that, under the conditions used here, the effect of extracellular pH established during the 30 min equilibration period, could not be reversed during glucose utilisation.

3. THE EFFECT OF EXTRACELLULAR pH UPON REGULATION OF GLUCOSE METABOLISM IN 20h CELLS UTILISING 50mM GLUCOSE

GLUCOSE UPTAKE GLYCOLYSIS AND POLYSACCHARIDE SYNTHESIS.

The amount of glucose taken up, ethanol formed and polysaccharide synthesised at 15 min, 30 min and 60 min after addition of 50mM glucose to cells suspended at pH 4.5 and pH 7.5 is shown in Table 7. Qualitatively the results were very similar to those obtained with 5mM glucose (see Fig 3). Thus both the rate of glucose uptake and glycolysis was reduced at pH 7.5 compared to pH 4.5, whilst the rate of polysaccharide synthesis was the same at the two pH values.

HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE. FIG 12.

After an initial overshoot a steady state level of HMP and FDP was established in which the intracellular content of HMP was highest at pH 7.5 whilst that of FDP was very similar at the two pH values. This higher intracellular content of HMP at pH 7.5 was associated with both a lower rate of glycolysis and glucose uptake (Table 7) compared to pH 4.5. Therefore the activity of phosphofructokinase was lower at pH 7.5 than pH 4.5 (Newsholme and Gevers, 1967) and it appeared that at both pH values phosphofructokinase controlled both the glycolysis and, via HMP, the glucose transport system (Sols, 1968).

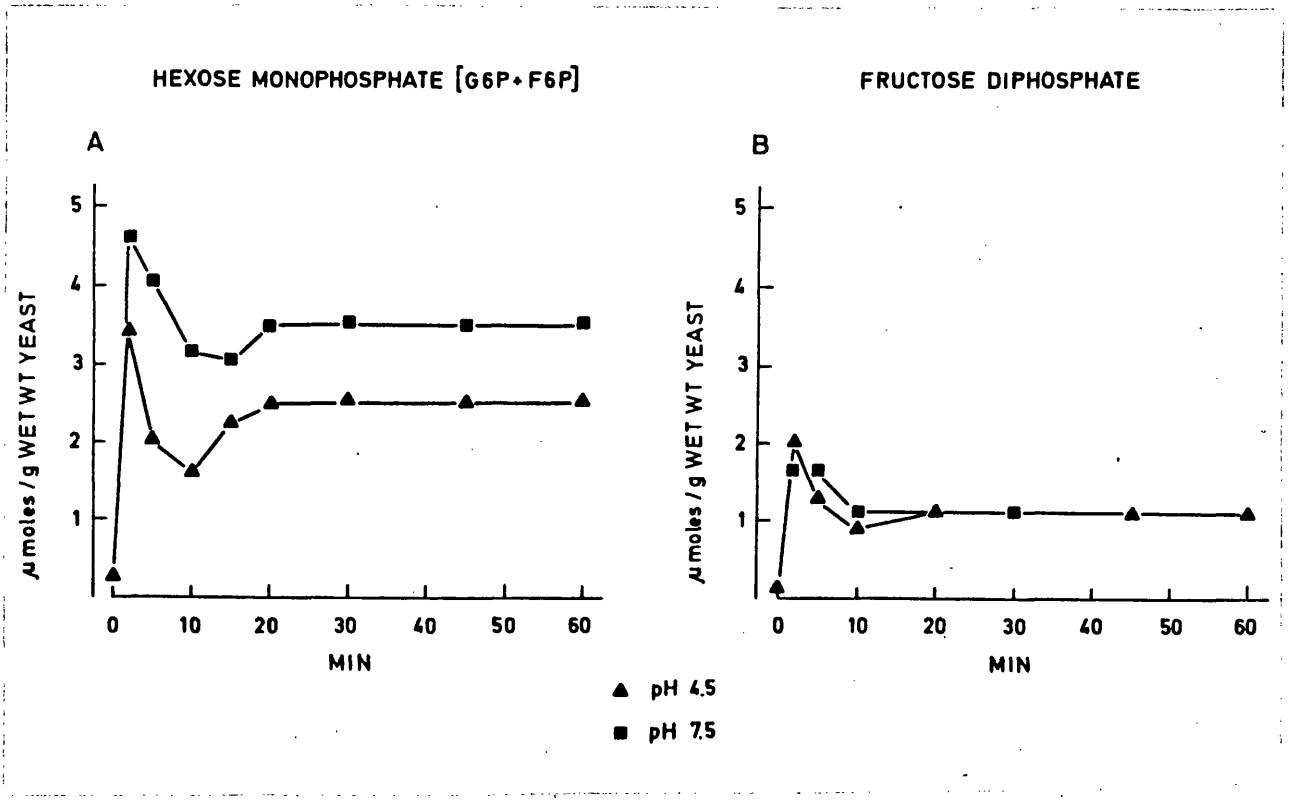
ADENINE NUCLEOTIDES AND CITRIC ACID.

The intracellular content of ATP, ADP and citric acid and the energy status (ATP/ADP) of the cells at 15 min, 30 min and 60 min after addition of glucose to the cells is shown in Table 8. With 50mM glucose little or no difference in the energy status of the cells was found between the two pH values. However in a single experiment the intracellular content of citric acid was found to be markedly

TABLE 7. THE AMOUNT OF GLUCOSE TAKEN UP ETHANOL PRODUCED AND POLYSACCHARIDE SYNTHESISED AT 15 MIN 30 MIN AND 60 MIN BY 20h CELLS (50mM GLUCOSE)

μmoles GLUCOSE EQUIVALENT PER g WET WT YEAST.	MIN	pH	
		4.5	7.5
Glucose taken up n = 3	15	412 ± 60	334 ± 25
	30	850 ± 27	694 ± 42
	60	1700 ± 50	1400 ± 70
Ethanol produced n = 3	15	136 ± 5	103 ± 6
	30	300 ± 20	233 ± 10
	60	650 ± 40	500 ± 58
Polysaccharide synthesised. n = 3	15	46 ± 3	57 ± 6
	30	105 ± 11	105 ± 5
	60	240 ± 24	248 ± 15

FIG 12 INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE AND FRUCTOSE DIPHOSPHATE (50 mM GLUCOSE)



for mean values ± SEM of HMP see APPENDIX 11.

TABLE 8. THE INTRACELLULAR CONTENT OF ADENINE NUCLEOTIDES AND CITRIC ACID AT 15 MIN 30 MIN AND 60 MIN IN 20h CELLS (50mM GLUCOSE)

μmoles PER g WET WT YEAST	MIN	pH 4.5	pH 7.5
Adenosine Triphosphate n = 4	15	2.7 ± 0.3	2.3 ± 0.1
	30	2.6 ± 0.3	2.3 ± 0.2
	60	2.5 ± 0.1	2.2 ± 0.2
Adenosine Diphosphate n = 4	15	0.9 ± 0.2	0.7 ± 0.1
	30	0.8 ± 0.1	0.7 ± 0.1
	60	0.8 ± 0.1	0.7 ± 0.1
Energy status (ATP/ADP)	15	3.0	3.3
	30	3.2	3.3
	60	3.1	3.1
Citric Acid n = 1	15	1.8	6.0
	30	3.6	11.4
	60	4.9	14.5

higher at pH 7.5 than at pH 4.5. Thus with cells metabolising 50mM glucose the lower activity of phosphofructokinase, at pH 7.5 compared with pH 4.5 appeared to be due to a higher intracellular content of citric acid (Salas et al, 1965).

SUMMARY. With cells metabolising both 5mM and 50mM glucose the activity of phosphofructokinase was lower at pH 7.5 than at pH 4.5. This lower activity of phosphofructokinase at pH 7.5 reduced the activity of the glycolysis system whilst that of the polysaccharide synthesising system remained unchanged compared to pH 4.5. Thus the relative activities of the polysaccharide synthesising and glycolysis systems were effectively altered by changes in the activity of phosphofructokinase. Although the rate of polysaccharide synthesis was the same at pH 4.5 and pH 7.5, with 5mM glucose more polysaccharide was eventually synthesised at pH 7.5 than at pH 4.5 (see Fig 3B). This was due to a lower rate of glucose uptake at pH 7.5 caused by the lower activity of phosphofructokinase, so that polysaccharide synthesis continued for a longer time at this pH value. This was also the case with 50mM glucose since, in a single experiment, more polysaccharide was synthesised from the available glucose (3750μmoles per g wet wt yeast) at pH 7.5 (840μmoles per g wet wt yeast) than at pH 4.5 (650μmoles per g wet wt yeast). Thus since less glucose was degraded via glycolysis and more synthesised to polysaccharide due to the lower activity of phosphofructokinase at pH 7.5 compared to pH 4.5, the activity of this enzyme clearly regulated the distribution of incorporated glucose between the polysaccharide synthesising and glycolysis systems under *in vivo* conditions in a fashion similar to that proposed from in *vitro* studies (Ramaiah, Hathaway and Atkinson, 1964).

4. CONTROL OF POLYSACCHARIDE SYNTHESIS

4. CONTROL OF POLYSACCHARIDE SYNTHESIS.

Rothman and Cabib (1967) reported that *in vitro* the glycogen synthetase from *S.cerevisiae* was activated by G6P and have shown that *in vivo* a decrease in the intracellular content of G6P resulted in a decrease in the rate of glycogen synthesis (Rothman and Cabib, 1969).

In the cells used here, during utilisation of 5mM glucose, the intracellular content of HMP and therefore G6P was higher at pH 7.5 than at pH 4.5 (see Fig 4A). However the rate of polysaccharide synthesis was the same at these two pH values (see Fig 3C) which indicated that the conditions and therefore the rate of polysaccharide synthesis might have already been optimal at pH 4.5, for the cells and conditions used here. To determine whether this was in fact the case, the rate of polysaccharide synthesis at pH 4.5 and pH 7.5 was measured with 50mM glucose as substrate. NB. The pH 2.0 condition need not be considered in this context since the effect of extracellular pH upon the glucose transport system, at this pH, reduced substrate supply to both the polysaccharide synthesising and glycolysis system. That the low rate of polysaccharide synthesis, at pH 2.0, was not due to an inhibition of glycogen synthetase was shown by measuring the intracellular content of UDPG at pH 2.0 and pH 4.5 during utilisation of 5mM glucose (Fig 13). It can be seen that UDPG did not accumulate at pH 2.0 whereas it did at pH 4.5. Also no accumulation of G1P was detected at pH 2.0 compared to pH 4.5.

In Table 9 the amount of polysaccharide synthesised at 15 min and 30 min after addition of 5mM and 50mM glucose to cells suspended at pH 4.5 and pH 7.5 is shown. It can be seen that the same rate of polysaccharide synthesis was achieved with both glucose concentrations,

during the 30 min period. This was in spite of the fact that more glucose was taken up (Table 9) and the intracellular content of HMP was higher (Fig 14) with 50mM compared to 5mM glucose, during the 30 min period. Therefore the rate of polysaccharide synthesis achieved at pH 4.5 with 5mM glucose was in fact maximal, under the conditions used, and it could not be expected that an increase in the intracellular content of G6P would increase the rate of polysaccharide synthesis.

STUDIES UPON A TRICHLOROACETIC ACID SOLUBLE POLYSACCHARIDE FRACTION.

It might be argued that the rates of glycogen synthesis could have been different in these cells but that synthesis of other polysaccharides masked the differences. Therefore during the early stages of this work the total amount of polysaccharide synthesised was routinely fractionated into a TCA insoluble (GLYCOGEN ? Trevelyan and Harrison, 1956) and a TCA soluble fraction (TREHALOSE ? Trevelyan and Harrison, 1952).

It was found that the amount of polysaccharide recovered in the TCA soluble fraction increased with increasing extracellular pH whereas the amount present in the TCA insoluble fraction was similar at the three pH values (Table 10).

Exhaustive TLC chromatography of the TCA soluble polysaccharide failed to identify trehalose. Instead the TCA soluble polysaccharide was found to consist of a mixture of slow moving components from which partial acid hydrolysis yielded a component chromatographically identical with maltose and complete acid hydrolysis yielded only glucose. Therefore the TCA soluble fraction appeared to consist of a mixture of relatively small molecular weight α 1-4 linked glucose polymers and not in this case trehalose. (Trevelyan and Harrison, 1952).

FIG 13 INTRACELLULAR CONTENT OF URIDINE DIPHOSPHOGLUCOSE DURING GLUCOSE UTILISATION (5 mM GLUCOSE)

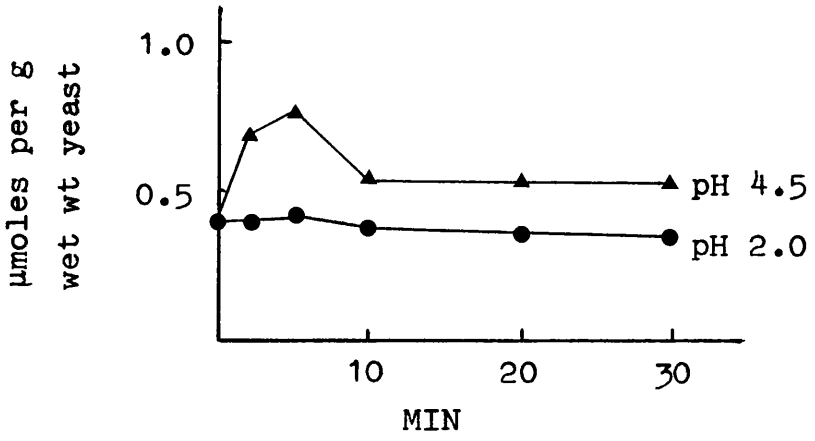
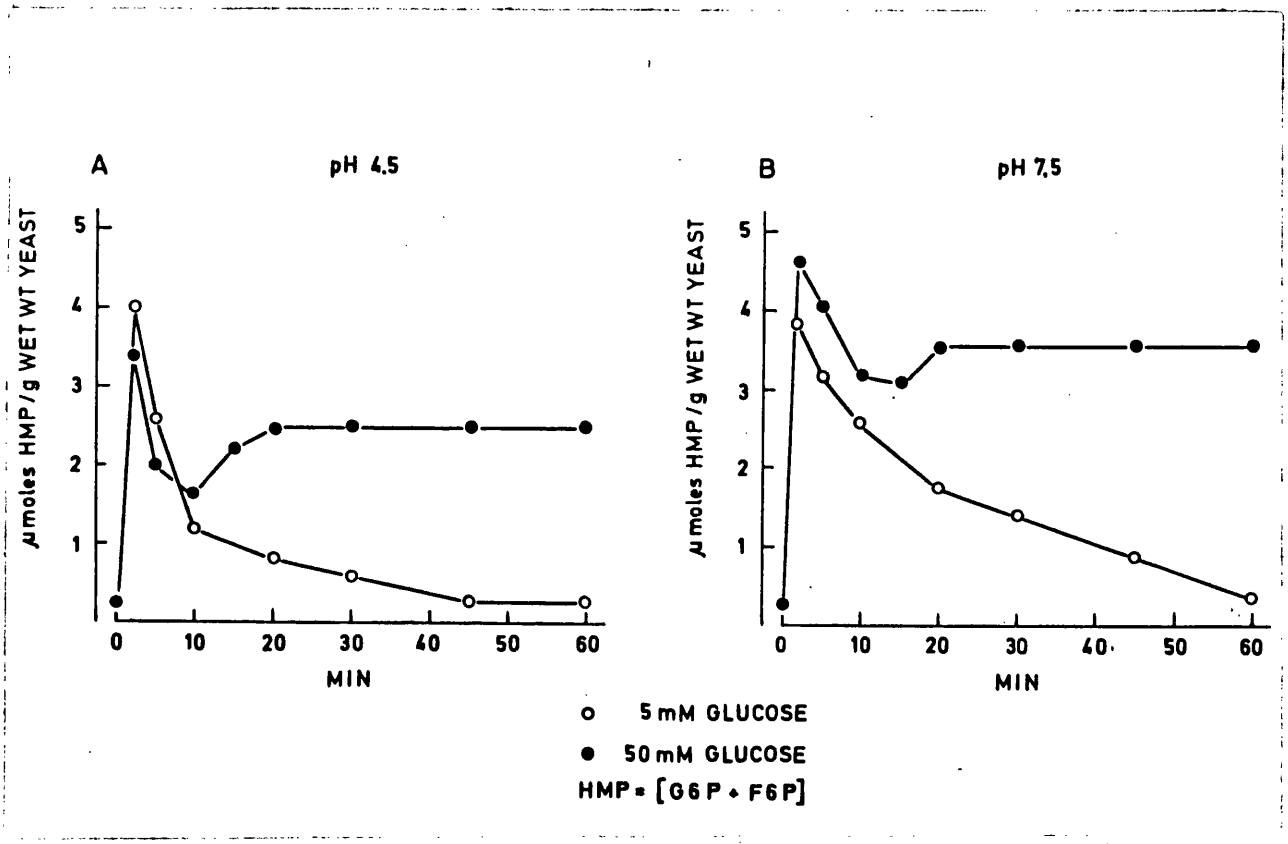


TABLE 9. THE AMOUNT OF POLYSACCHARIDE SYNTHESISED AND GLUCOSE TAKEN UP AT 15 MIN AND 30 MIN BY 20h CELLS WITH 5mM + 50mM GLUCOSE.

μ moles PER g WET WT YEAST	MIN	GLUCOSE mM	pH 4.5	pH 7.5
	15	5	55 ± 10	53 ± 8
	15	50	46 ± 3	57 ± 6
Polysaccharide	30	5	108 ± 14	110 ± 2
synthesised	30	50	105 ± 11	105 ± 5
n = 3				
	15	5	265*	185*
	15	50	412 ± 60	334 ± 25
Glucose taken up				
5mM n = 5	30	5	371 ± 2	330 ± 6
50mM n = 3	30	50	850 ± 27	694 ± 42

* Value obtained from glucose uptake curve (cf FIG 2A)

FIG 14 THE INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE WITH 5 mM and 50 mM GLUCOSE



see APPENDIX for mean values ± SEM:
5 mM GLUCOSE pH 4.5 APP 5. pH 7.5 APP 6.
50 mM GLUCOSE APP 11.

TABLE 10 THE AMOUNT OF TRICHLOROACETIC ACID SOLUBLE AND INSOLUBLE POLYSACCHARIDE SYNTHESISED BY 18h CELLS (5mM GLUCOSE).

μmoles GLUCOSE EQUIVALENT PER g WET WT YEAST	pH		
	2.0	4.5	7.5
TCA Soluble Polysaccharide n = 5	0	37 ± 1	61 ± 1
TCA Insoluble Polysaccharide n = 5	30 ± 2	47 ± 3	43 ± 1
Total Polysaccharide n = 5	30 ± 2	84 ± 3	104 ± 3

These results suggested that changes in extracellular pH effected the synthesis of the TCA soluble fraction whilst that of the TCA insoluble fraction was largely unaffected. However it was subsequently found that the TCA soluble polysaccharide was a degradation product formed during the extraction procedure. This appeared to be the case since the amount recovered increased with increasing time of extraction of the cells. Therefore fractionation of polysaccharide was discontinued and only the total amount of polysaccharide synthesised was measured.

It is likely that the increase in polysaccharide measured in this work was largely due to glycogen synthesis. This can be deduced from the results presented by Rothman and Cabib (1969) who measured glycogen by a specific enzymic method. Thus it can be calculated from their results that under very similar conditions to those used here (i.e. 1.3g compared to 1.2 g wet wt yeast per 100 ml buffer, pH 5.5 compared to pH 4.5 and 75mM compared to 50mM glucose), the amount of glycogen synthesised was very similar to the total amount of polysaccharide synthesised in a single experiment in this work. (ie Ca 550μmoles glycogen compared to 640μmoles total polysaccharide per g wet wt yeast). The fact that less polysaccharide was synthesised from a higher glucose concentration in their (Rothman and Cabib , 1969) work can be explained by the essentially anaerobic conditions used whereas aerobic conditions were used here. Thus resting cells of S. cerevisiae are known to synthesis less polysaccharide under anaerobic conditions than aerobic conditions (Rothstein and Enns, 1946).

A P P E N D I X

Appendix 1

5 mM GLUCOSE. ETHANOL AND RESPIRATION

Min.	pH 2.0				pH 4.5				pH 7.5			
	Ethanol		Respiration		Ethanol		Respiration		Ethanol		Respiration	
	mean	sem.	mean	sem.	mean	sem.	mean	sem.	mean	sem.	mean	sem.
5	23	2	7	1	45	4	11	2	12	1	8	2
10	52	6	13	2	75	4	22	2	27	3	17	4
20	106	14	26	4	89	11	43	3	27	3	35	5
30	156	13	39	2	64	15	68	4	23	2	50	4
60	145	10	78	5	0		130	6	0		88	10
120	67	10	156	7	-		130	6	-		88	10
180	0		225	5	-		-		-		-	

n = 4 - Ethanol

n = 3 - Respiration

+ µmoles glucose equivalent per g wet wt yeast

Appendix 2

5 mM GLUCOSE. GLUCOSE/ 75 ml

Min	pH 2.0		pH 4.5		pH 7.5	
	mean	sem.	mean	sem.	mean	sem.
	+					
0	375		375		375	
2	343	10	303	9	326	16
5	309	7	263	2	286	12
10	255	10	176	4	244	15
20	159	10	36	9	128	11
30	61	6	0		45	6
60	0		-		0	

n = 4

+ µmoles per 75 ml buffer (1 g wet wt yeast per 75 ml).

Appendix 3

5 mM GLUCOSE. POLYSACCHARIDE SYNTHESIS

Min	pH 2.0		pH 4.5		pH 7.5	
	mean	sem.	mean	sem.	mean	sem.
			+			
15	-		55	10	53	8
30	42	6	108	14	110	2
60	63	5	110	10	157	5
120	63	5	110	10	157	5

n = 6

+ µmoles glucose equivalent per g wet wt yeast.

Appendix 4

pH 2.0 5mM GLUCOSE. INTRACELLULAR CONTENT GLYCOLYSIS INTERMEDIATES

	HMP (G6P+F6P)		FDP		TP (DHAP+GAP)		PGA (3PG+2PG)		PEP	
Min	n = 4 mean	sem.	n = 4 mean	sem.	n = 4 mean	sem.	n = 2 mean	sem.	n = 2 mean	sem.
0	0.14	0.14	0.31	0.10	0.19	0.02	1.25		0.35	
2	2.20	0.17	1.80	0.10	0.64	0.17	0.65		0.10	
5	1.64	0.08	2.17	0.08	0.70	0.10	0.70		0.10	
10	1.20	0.08	2.28	0.12	0.75	0.10	0.60		0.10	
20	1.00	0.10	1.90	0.28	0.60	0.17	0.65		0.10	
30	0.75	0.10	1.70	0.36	0.50	0.14	0.65		0.10	
45	0.45	0.10	1.40	0.20	0.35	0.10	0.70		0.10	
60	0.25	0.15	1.10	0.10	0.25	0.10	0.65		0.10	

+ µmoles per g wet wt yeast

Appendix 5

pH 4.5 5 mM GLUCOSE. INTRACELLULAR CONTENT GLYCOLYSIS INTERMEDIATES

	HMP (G6P+F6P)		FDP		TP (DHAP+GAP)		PGA (3PG+2PG)		PEP	
Min	n = 4 mean	sem.	n = 4 mean	sem.	n = 4 mean	sem.	n = 2 mean	sem.	n = 2 mean	sem.
	+									
0	0.26	0.07	0.09	0.10	0.12	0.01	2.49		0.80	
2	4.00	0.10	2.25	0.08	0.72	0.01	0.70		0.10	
5	2.60	0.20	1.55	0.20	0.50	0.01	0.70		0.10	
10	1.20	0.03	0.60	0.06	0.40	0.06	0.90		0.20	
20	0.80	0.03	0.25	0.03	0.30	0.04	1.70		0.50	
30	0.60	0.05	0.20	0.06	0.23	0.04	2.52		0.70	
45	0.25	0.10	0.15	0.10	0.10	0.10	2.50		0.70	
60	0.25	0.15	0.15	0.05	0.15	0.10	2.51		0.75	

+ µmoles per g wet wt yeast

Appendix 6

pH 7.5 5 mM GLUCOSE. INTRACELLULAR CONTENT OF GLYCOLYSIS INTERMEDIATES

	HMP (G6P+F6P)		FDP		TP (DHAP+GAP)		PGA (3PG+2PG)		PEP	
Min	n = 4 mean	sem.	n = 4 mean	sem.	n = 4 mean	sem.	n = 2 mean	sem.	n = 2 mean	sem.
	+									
0	0.30	0.01	0.11	0.05	0.09	0.07	2.78		0.88	
2	3.80	0.15	1.44	0.14	0.41	0.01	0.70		0.10	
5	3.15	0.10	1.64	0.20	0.50	0.01	0.70		0.10	
10	2.50	0.10	0.60	0.14	0.30	0.02	1.10		0.25	
20	1.75	0.10	0.47	0.10	0.25	0.02	1.70		0.40	
30	1.42	0.10	0.32	0.07	0.20	0.10	1.95		0.50	
45	0.90	0.10	0.20	0.10	0.10	0.05	2.80		0.80	
60	0.40	0.10	0.10	0.05	0.10	0.07	2.85		0.82	

+ µmoles per g wet wt yeast

Appendix 7

pH 2.0 5 mM GLUCOSE. INTRACELLULAR CONTENT OF ADENINE NUCLEOTIDES

Min	ATP mean	sem.	ADP mean	sem.	AMP
	+				
0	2.10	0.10	1.40	0.20	-
2	2.00	0.10	1.47	0.10	-
5	1.90	0.10	1.60	0.06	0.07
10	1.95	0.06	1.52	0.05	0.10
20	1.85	0.01	1.35	0.07	0.15
30	1.80	0.01	1.35	0.10	0.22
45	1.67	0.05	1.45	0.10	0.25
60	1.55	0.01	1.60	0.10	0.32

n = 4

+ µmoles per g wet wt yeast

Appendix 8

pH 4.5 5 mM GLUCOSE. INTRACELLULAR CONTENT OF ADENINE NUCLEOTIDES

Min	ATP		ADP	
	mean	sem.	mean	sem.
	+			
0	2.46	0.10	1.00	0.17
2	2.40	0.10	1.00	0.16
5	2.22	0.10	0.90	0.17
10	2.25	0.10	0.80	0.17
20	2.30	0.15	0.65	0.10
30	2.35	0.17	0.60	0.06
45	2.57	0.10	0.60	0.10
60	2.90	0.15	0.55	0.05

n = 4

+ µmoles per g wet wt yeast

Appendix 9

pH 7.5 5 mM GLUCOSE. INTRACELLULAR CONTENT OF ADENINE NUCLEOTIDES

Min	ATP mean	sem.	ADP mean	sem.
	+			
0	2.35	0.10	0.75	0.14
2	2.30	0.10	0.80	0.20
5	2.35	0.06	0.72	0.10
10	2.38	0.12	0.62	0.10
20	2.45	0.16	0.48	0.14
30	2.55	0.07	0.42	0.10
45	2.70	0.10	0.35	0.10
60	2.77	0.10	0.30	0.12

n = 4

+
$\surd$umoles per g wet wt yeast

Appendix 10

5 mM GLUCOSE. INTRACELLULAR CONTENT OF CITRIC ACID

Min	pH 2.0 mean	sem.	4.5 mean	sem.	7.5 mean	sem.
	+					
0	0.45	0.15	1.40	0.14	1.73	0.14
2	0.45	0.14	1.30	0.20	2.10	0.20
5	0.52	0.07	1.55	0.20	1.90	0.30
10	0.57	0.11	2.15	0.17	1.95	0.30
20	0.76	0.14	2.30	0.20	2.70	0.20
30	0.75	0.05	2.50	0.20	3.30	0.17
45	0.70	0.10	2.90	0.18	3.50	0.10
60	0.60	0.10	3.35	0.20	3.70	0.20

n = 4

+ μmoles per g wet wt yeast

Appendix 11

pH 4.5 and pH 7.5 50 mM GLUCOSE. INTRACELLULAR CONTENT OF HEXOSE MONOPHOSPHATE

Min	pH 4.5 mean	sem.	pH 7.5 mean	sem.
	+			
15	2.25	0.22	3.10	0.20
30	2.50	0.20	3.50	0.20
60	2.50	0.31	3.50	0.15

n = 3

+ µmoles per g wet wt yeast

D I S C U S S I O N

The interpretation of these in vivo results depended upon the use of several assumptions. The assumptions used and the findings based upon them were:

1. If the intracellular content of the substrate, but not the product, of an enzyme within a pathway changes in such a manner that differences between two or more conditions, or changes within a single condition, are inversely related to changes in the rate of flux along that pathway, that enzyme is regulatory (Newsholme and Gevers, 1967). Judged by this criteria both phosphofructokinase and pyruvate kinase were shown to be regulatory enzymes within the glycolysis system, under the conditions used here.

2. If the intracellular content of the substrate, but not the product, of only one enzyme within a pathway is, in two or more conditions, inversely related to differences in the rate of flux along that pathway, that enzyme regulates the overall rate of flux along the pathway (Newsholme and Gevers, 1967). Judged by this criteria phosphofructokinase was shown to regulate the activity of the glycolysis system, in cells suspended at each pH value, under the conditions used here.

3. The glucose transport system of S.cerevisiae is feed back regulated by hexosemonophosphate (Sols 1968). Thus the activity of the glucose transport system can be said to be regulated by the intracellular content of HMP, when under two or more conditions the rate of glucose uptake is inversely related to the intracellular content of HMP (Sols, 1968).

   Judged by this criteria the activity of the glucose transport system was regulated by the intracellular content of HMP in cells suspended at pH 4.5 and pH 7.5, [redacted] At pH 2.0 [redacted] this was not the case.

[redacted]. In addition since phosphofructokinase regulated the intracellular content of HMP, at pH 4.5 and pH 7.5, it could be said that the activity of this enzyme regulated the activity of the glucose transport system as well as the activity of the glycolysis system at these two pH values.

4. That *in vitro* findings may be translated to the *in vivo* condition. Clearly such an assumption first depends upon demonstrating a similar relationship between an enzyme and its effectors under both *in vitro* and *in vivo* conditions. Judged by this criteria FDP was shown to regulate changes in the activity of pyruvate kinase in the cells used here.

The primary objective of the investigations reported in this thesis was to determine why, with increasing extracellular pH, the amount of glucose synthesised to polysaccharide increased, whereas the amount respired decreased. This objective appears to have been largely reached and the phenomenon may be attributed to an effect of extracellular pH which changed the activity of phosphofructokinase, together with an additional effect upon the activity of the glucose transport system in cells suspended at pH 2.0. The evidence and arguments upon which this conclusion was based have been presented and discussed in the results section and therefore will not be repeated here. However the overall findings will be summarised.

At pH 2.0 a direct effect of extracellular pH reduced the activity of the glucose transport system thereby limiting the supply of substrate to both the glycolysis and polysaccharide synthesising systems. Due to a high activity of phosphofructokinase in cells suspended at pH 2.0, a high proportion of the incorporated glucose was degraded, via glycolysis, at any one time. This further limited the supply of

substrate to the polysaccharide synthesising system. This relationship was maintained throughout the course of glucose uptake so that more of the available glucose was degraded and less synthesised to polysaccharide, compared to pH 4.5 and pH 7.5.

At pH 4.5 and pH 7.5 the activity of the glucose transport system did not appear to be effected by extracellular pH. However due to a lower activity of phosphofructokinase at pH 7.5, the activity of both the glycolysis and glucose transport systems was reduced compared to pH 4.5, but that of the polysaccharide synthesising system was the same at the two pH values. Thus at pH 7.5 a lower activity of phosphofructokinase than at pH 4.5 reduced the activity of the glycolysis system in relation to that of the polysaccharide synthesising system. This relationship was maintained throughout the course of glucose uptake, and the lower rate of glucose uptake at pH 7.5, itself controlled by phosphofructokinase, allowed polysaccharide synthesis to continue for a longer time than at pH 4.5. Thus more polysaccharide was eventually synthesised, from the available glucose, at pH 7.5 as well as less being degraded, due to the low activity of phosphofructokinase in cells suspended at this pH value.

It could be concluded then that the activity of phosphofructokinase, under these *in vivo* conditions, regulated the distribution of incorporated glucose between the polysaccharide synthesising and glycolysis systems as was proposed, from *in vitro* studies upon the enzyme, by Ramaiah, Hathaway and Atkinson (1964).

The fact that the amount of glucose respired decreased with increasing extracellular pH was explained by the finding that ethanol produced during glucose utilisation, the amount of which decreased with increasing extracellular pH, was subsequently oxidised completely.

Differences in the energy status and citric acid content of the cells, which appeared to be the cause of the differences in the activity of phosphofructokinase between cells suspended at the three pH values, were not included in the above explanation. This was because certain considerations, which follow here, suggest that possible changes in the intracellular pH of the cells could contribute towards the observed differences in the activity of phosphofructokinase.

Differences in the energy status and citric acid content of the cells suspended at the three pH values were established prior to the addition of glucose to the cells. These differences could conceivably have been established for the following reason: Both H^+ and Na^+ions, which were the only cations originally present in the sodium phosphate buffers used in these investigations, compete for sites of a cation transport system present in S.cerevisiae (Rothstein, 1959). Thus since the ratio of H^+/Na^+ion increased with decreasing pH, H^+ion should be increasingly transported into the cell with decreasing extracellular pH. Therefore it is possible that an increasing energy demand, caused by the necessity to actively extrude such H^+ion from the cell caused the decreasing energy status of the cells observed with decreasing pH in the resting condition. By such a mechanism the energy status of the cells might be effected without any appreciable change in the intracellular pH of the cells.

This alteration in the energy status of the cells in turn would be expected to alter the intracellular content of citric acid in the observed manner as a consequence of the sensitivity of isocitric dehydrogenase to changes in energy status (Hathaway and Atkinson, 1963).

However a substantial effect of extracellular pH upon the intracellular pH of resting cells of S.cerevisiae has been reported

(Kotyk, 1963). Therefore the possibility that a similar effect might have occurred under the conditions used in this investigation must be considered.

Several deductions in relation to the extent and effect of any change in the intracellular pH of the cells used here can be made from the results presented here. Firstly, that any change must have been within what may be termed the "physiological range" since metabolism was not inhibited completely at any pH value and in fact the activity of enzymes of the glycolysis system in cells suspended at pH 2.0 was at least equal to that in cells suspended at pH 7.5. Secondly, any changes which had occurred could not have seriously effected glycolytic enzymes other than phosphofructokinase, since all the evidence indicated that this enzyme was the rate controlling step in the glycolysis sequence at each of the three pH values. Therefore the effect of any change in intracellular pH need be considered in relation to that effect upon only the activity of phosphofructokinase.

In order to consider seriously the effect of changes in the intracellular pH of the cells it must be assumed that a change of extracellular pH in one direction would cause a change of intracellular pH in the same direction (Kotyk, 1963). Also there seems to be little point in considering only hypothetical changes in intracellular pH of unknown degree and range with reported pH - activity curves for phosphofructokinase from S. cerevisiae. Given this assumption it is possible to consider the effect of a change of intracellular pH, which decreases with decreasing extracellular pH, in relation to its effect upon the sensitivity of phosphofructokinase to the adenine nucleotide ATP. This is because reports are present in the literature concerning the effect of pH upon inhibition of phosphofructokinase

from S.cerevisiae by ATP (Lindell and Stellwagen, 1968; Kopperschlager et al, 1968; Atzpodien and Bode, 1970).

Thus it has been reported that the inhibitory effect of ATP upon the phosphofructokinase of S.cerevisiae, decreases with decreasing pH (Lindell and Stellwagen, 1968; Kopperschlager et al, 1968; Atzpodien and Bode, 1970). This appears to be due to a decrease in the affinity of ATP for an inhibitor site and an increase in its affinity for the catalytic site (Kopperschlager et al, 1968). Therefore it might be argued that a combination of a low inhibitory effect of ATP together with the stimulatory effect of a low ATP and high AMP level on phosphofructokinase (Ramaiah, Hathaway and Atkinson, 1964; Betz and Moore, 1967) would counteract any effect of a reduced intracellular pH, in cells suspended at pH 2.0, and result in the high activity of phosphofructokinase observed in cells suspended at this pH value. Again, an increased inhibitory effect of ATP, due to an increased intracellular pH, in cells suspended at pH 7.5 might be expected to increase the inhibitory effect of a higher ATP level (20h cells, 5mM glucose, Fig 7) and might even lead to a lower activity of phosphofructokinase with a lower (18h cells, 5mM glucose, Table 3) or similar (20h cells, 50mM glucose, Table 8) level of ATP, compared to cells suspended at pH 4.5.

These considerations suggest that, if the intracellular pH of the cells used in the investigations reported here was effected by changes in extracellular pH, then the effect of such changes would contribute towards the differences in the activity of phosphofructokinase observed in cells suspended over the pH range from pH 2.0 to pH 7.5.

In addition to demonstrating that changes in an enviromental factor can effect regulatory processes inside the cells, the results presented in this thesis demonstrate the central role that the enzyme

phosphofructokinase plays in the regulation of aerobic glucose metabolism in resting cells of S.cerevisiae.

Thus evidence has been obtained to show that in a single intact cellular system phosphofructokinase may regulate the activity of the glycolysis and glucose transport system, the relative activities of the polysaccharide synthesising and glycolysis systems and hence the distribution of glucose between these two systems, as well as coordinating the activities of enzymes within the glycolysis system. These functions of phosphofructokinase have been demonstrated in vivo for single systems viz glycolysis (Lynen et al, 1959; Salas et al, 1955), glucose transport (Sols, 1968), polysaccharide synthesis (Rothman and Cabib, 1969) and have been proposed from in vitro studies upon isolated enzymes viz the distribution of glucose between the polysaccharide synthesising and glycolysis systems (Ramaiah, Hathaway and Atkinson, 1964; Rothman and Cabib, 1967), coordination of the activities of enzymes in the glycolysis system ie phosphofructokinase itself and pyruvate kinase (Hess, Haeckel and Brand, 1966; Haeckel et al 1968). However, there does not appear to be any report in the literature concerning these multiple functions of this key regulatory enzyme in intact yeast cells.

It has also been proposed from in vitro studies that the activity of phosphofructokinase and hence the metabolic fate of incorporated glucose would be regulated in vivo by the energy status and therefore the energy requirements of the yeast cell (Ramaiah, Hathaway and Atkinson, 1964). Due to the possible effect of a changed intracellular pH upon the activity of phosphofructokinase it cannot be said that the differences in its activity in 20h cells utilising 5mM glucose was due solely to differences in the energy status of the

cells. However it appears possible that these differences in the energy status of the cells, at least in part, could have regulated the activity of phosphofructokinase to the extent that these differences were maintained and even increased during glucose utilisation, whereas had they been reversed it is likely that the activity of phosphofructokinase and therefore the metabolic fate of glucose, at any one pH value, would have been altered.

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